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THE EXCRETION OF CREATINE AND CREATININE PARENTERALLY INTRODUCED.

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(Received for publication, December 6, 1916.)

The experiments of Folin (1) in 1906 led him to doubt that any metabolic relation exists between creatine and creatinine and for a time suppressed the principle laid down by Liebig (2) and generally accepted that urinary creatinine results from the metabolism of muscle creatine.

All experiments on the metabolism of creatine and creatinine in which the substances are fed are open to the criticism that absorption of the unchanged substances may never occur because of their destruction by microorganisms in the alimentary tract. Mellanby (3) in 1908 applied this criticism to Folin's experiment mentioned above, and in 1912 Twort and Mellanby (4) isolated from the feces an organism capable of decomposing creatine.

The results of experiments in which creatine or creatinine have been introduced parenterally are somewhat conflicting. Lefmann (5) obtained no evidence that creatine is converted to creatinine. Pekelharing and Van Hoogenhuyze (6) and Myers and Fine (7) working with rabbits noted an increased elimination of creatinine after the injection of creatine, while a part of the creatine reappeared unchanged after the administration of comparatively large amounts.

That creatine ingestion may be followed by increased creatinine excretion is further confirmed by several investigators, especially by Rose and Dimmitt (8) who fed large amounts, up to 20 gm. per day, of creatine to men. They obtained an unmistakable increase in creatinine output and recovered, unchanged in the urine, a part of the ingested creatine, as high as 75 per cent when the largest amounts were fed. After feeding creatinine, up to 16 gm. per day, 77 to 78 per cent was recovered in the urine unchanged while no creatine was excreted. These results lead Myers and Fine, and Rose and Dimmitt to conclude that an intimate physiological relation exists between creatine and creatinine.

The results of our experiments support this view. In order to avoid danger of bacterial decomposition of the materials in the intestine we injected sterilized solutions of creatine and creatinine subcutaneously. The 24 hour urines were analyzed daily by

the following methods: creatinine by Folin's method (9); creatine by the Folin-Benedict method (10); ammonia by Folin's method (11); urea by the method of Van Slyke and Cullen (12); total nitrogen by the Kjeldahl method.

The creatine used was prepared partly from dog muscle and partly from python muscle, the creatinine from urine by Benedict's method (13). The purity of the specimens was established by their nitrogen content. The creatine and creatinine were dissolved in 0.9 per cent sodium chloride solution and sterilized by boiling. The creatine solution after sterilizing gave a negative test for creatinine.

Experiments with Man.

A normal man, 28 years of age, weighing 80 kilos, and 1.8 meters in height served as subject. The diet was creatine-creatinine-free and consisted of bread, butter, sugar, starch pudding, chocolate, vegetables, and fruit. The character of the diet was constant but the quantity varied somewhat from day to day. The first injection was made on the 5th day after beginning the diet when the elimination of creatinine had become constant. The injections were made in the thigh or calf, the creatinine at one dose, and the creatine divided and given in thirteen portions at intervals of 30 minutes until all had been taken. Analyses of the first 4 days' urines are not included. The results follow.

Effect of Subcutaneous Injections of Creatine and Creatinine on Their Elimination in the Urine.

Day.	Volume.	Reaction to litmus.	Sp. gr.	Total N.	Urea N.	Ammonia N.	Creatinine N.	Creatine N.	Injected nitrogen recovered. As creatine.	Injected nitrogen recovered. As creatinine.
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
5	560	Acid.	1.025	5.81	4.50	0.43	0.52	0.000		
6	500	"	1.029	6.11	4.57	0.47	0.64	0.024	4	20
7	600	"	1.020	5.61	4.20	0.41	0.52	0.000		
8	576	Neutral.	1.023	6.53	5.16	0.46	0.56	0.0077	4	19
9	640	Acid.	1.020	5.88	4.59	0.41	0.52	0.000		
10	592	"	1.024	6.09	4.46	0.50	0.76	0.000	0	66

On the 6th day injected creatine containing 0.584 gm. of N.
" " 8th " " " 0.194 " " "
" creatinine " 0.361 " " "

The fact that 19 to 20 per cent of the injected creatine nitrogen was recovered in the urine as creatinine is evidence that an intravital transformation of creatine to creatinine can occur. Since only 4 per cent of the injected creatine was excreted unchanged in the urine, 76 to 77 per cent of the injected nitrogen remains to be accounted for. Slight increases in total nitrogen, urea, and ammonia eliminated suggest a decomposition of the creatine or its transformation product creatinine. Our data on this point are not conclusive since there was a slight variation in the amount of protein ingested from day to day. Gottlieb and Stangassinger (14) have demonstrated the presence of creatine- and creatinine-destroying enzymes in autolyzing tissues, and Benedict and Osterberg (15), among others, have expressed the view that creatine may be destroyed in relatively large amounts in the normal mammal. That a part of the injected creatine was stored in the muscles is possible since Myers and Fine, and Folin and Denis (16) have noted an increase in muscle creatine after injection or absorption from the intestine of the same.

A large part of the injected creatinine (66 per cent) was recovered unchanged in the urine, without an increased creatine elimination. Rose and Dimmitt have argued that the creatine-creatinine transformation is not reversible in the body since creatine does not appear in the urine after ingestion of creatinine. This conclusion seems unwarranted to us since a small amount of creatine so formed might be completely destroyed or stored in the body. Myers and Fine have observed that the creatine content of rabbit muscle is increased by injection of creatinine into the intact animal.

Experiments with Rabbits.

Creatine and creatinine solutions were injected subcutaneously into rabbits as in the preceding experiment except that the creatine was given in a single dose instead of being divided into portions given at intervals. The results were substantially the same as with man. With the comparatively larger doses of creatine more was excreted unchanged and less converted into creatinine. The tables follow.

Rabbit I, Weight 1,980 Gm. Diet 300 Gm. of Carrots and 5 Gm. of Oats Daily.

Day.	Volume.	Reaction to litmus.	Sp. gr.	Total N.	Urea N.	Ammonia N.	Creatinine N.	Creatine N.	Injected nitrogen recovered. As creatine.	Injected nitrogen recovered. As creatinine.
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	180	Alkaline.	1.018	0.840	0.699	0.001	0.0240	0.000		
2	102	Acid.	1.028	0.720	0.561	0.003	0.0242	0.000		
3	96	Neutral.	1.026	0.701	0.548	0.001	0.0239	0.000		
4	150	Alkaline.	1.021	0.871	0.681	0.003	0.0473	0.000	0	77
5	120	"	1.020	0.699	0.502	0.000	0.0238	0.000		
6	101	"	1.029	0.740	0.560	0.007	0.0275	0.0156	51	11
7	150	"	1.025	0.816	0.642	0.004	0.0280	0.0129	43	13
8	190	"	1.012	0.806	0.630	0.004	0.0243	0.000		

On the 4th day injected creatinine containing 0.0297 gm. of N.
" " 6th " " creatine " 0.0302 " " "
" " 7th " " " " 0.0300 " " "

[illegible]t II, Weight 1,810 Gm. Diet 300 Gm. of Carrots and 5 Gm. of Oats Daily.

Day.	Volume.	Reaction to litmus.	Sp. gr.	Total N.	Urea N.	Ammonia N.	Creatinine N.	Creatine N.	Injected nitrogen recovered. As creatine.	Injected nitrogen recovered. As creatinine.
	cc.			gm.	gm.	gm.	gm.	gm.	per cent	per cent
1	160	Alkaline.	1.012	0.621	0.468	0.002	0.0244	0.000		
2	86	"	1.031	0.704	0.562	0.006	0.0234	0.000		
3	120	"	1.022	0.699	0.525	0.001	0.0230	0.000		
4	90	"	1.039	0.816	0.650	0.003	0.0460	0.000	0	79
5	130	Neutral.	1.016	0.760	0.545	0.004	0.0239	0.000		
6	96	Alkaline.	1.040	0.806	0.643	0.001	0.0268	0.0167	59	11
7	83	"	1.036	0.672	0.516	0.003	0.0270	0.0189	65	12
8	102	"	1.024	0.810	0.639	0.000	0.0236	0.000		

On the 4th day injected creatinine containing 0.0284 gm. of N.
" " 6th " " creatine " 0.0283 " " "
" " 7th " " " " 0.0288 " " "

SUMMARY.

Subcutaneous injections of creatine into rabbits and man were followed by increased urinary creatinine excretion which is further evidence of an intravital transformation of creatine to creatinine.

In man the larger part (76 to 77 per cent) of the creatine injected was destroyed or stored in the body.

The injection of creatinine was not followed by the excretion of creatine in the urine. This is not proof, however, that the creatine-creatinine mechanism is non-reversible.

Since writing the above a paper by Burns and Orr (17) has appeared in which they report no increased creatinine excretion after the ingestion of 0.5 gm. of creatine per day. They suggest that the increased creatinine elimination observed by Myers and Fine after the ingestion of creatine may have been due to a diuresis on the experimental days. In our experiments with man this criticism will not apply since the urine volume is no greater on the creatine than on the normal days.

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CHOLESTEROL AND CHOLESTEROL ESTERS IN HUMAN BLOOD.*

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(Received for publication, December 18, 1916.)

Cholesterol occurs in practically all animal tissues and is therefore probably an important substance in the animal economy. It was one of the earliest biochemical substances discovered and because of its wide occurrence and also its relative stability it has been the subject of much investigation. Nevertheless there is little definite knowledge regarding its origin, fate, or function in living beings.

Cholesterol originates in the animal body mainly from the food, either as such in animal food, or in plant material as isomeric forms which the animal is able to change into cholesterol.[1] There is no satisfactory agreement among investigators as to whether it can be synthesized in the body. Dezani[2] has claimed that it is readily synthesized, while Gardner and Lander[3] consider its synthesis doubtful. Lifschütz[4] believes that it is formed from oleic acid. It seems likely, at any rate, that although synthesis is probable it is not an important factor in the supply to the animal. Cholesterol is absorbed from the intestine, increasing the blood cholesterol,[5-8] and during absorption is partly esterified with the fatty acids, the extent of combination being quite definite.[9]

As regards its fate in the body, there is some evidence that it may be burned[10] and also that it may be a source of the bile acids.[11] Ordinarily, however, the greater part, if not all, of the excess of cholesterol is excreted in the feces or by the skin after partly undergoing slight oxidation or reduction. It is excreted in the intestine practically entirely free, while in the skin secretions it appears almost entirely as esters of the fatty acids. In certain animals, notably the rabbit, there appears to be a limit to the power of excretion, and if large amounts are fed, it accumulates first in the blood, then in the organs such as the suprarenals, liver, and

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* A report of this work was presented before the American Chemical Society in New York, September, 1916.

spleen, and also in the walls of the arteries, mainly as cholesterol esters, forming the anisotropic fat deposits.[5,6] Similar pathological deposits occur in human beings and there is an increasing tendency among clinicians to refer these and certain possibly resulting phenomena such as arteriosclerosis to excessive cholesterol in the diet. If cholesterol is excluded from the diet none is excreted, the organism apparently conserving its supply.[12]

The functions of cholesterol in the living organism are little understood. The one most widely discussed is that connected with the protective mechanisms of the body and particularly with its power to act as an antihemolytic against such substances as saponin,[13] tetanolysin,[14] etc. The free cholesterol alone is effective in this reaction, the esters having no antihemolytic power,[15] the reason being that the cholesterol probably forms an ester-like compound with the poison through the free hydroxyl group. Lecithin has been found to act antagonistically to cholesterol in this function by activating the lysin.[16] Cholesterol has been found by Robertson[17] to accelerate the growth of tumors in mice and also to stimulate the growth of rats,[18] in both of which lecithin has been found to have the opposite effect although to a much less degree. It has been found by Wacker[29] that cholesterol accumulates in the body fat with increasing age, and Robertson[30] has advanced the view, which is shared by other recent workers,[31] that this accumulation of cholesterol may account for the increased incidence of tumors with advancing age. A third function of cholesterol and one to which little attention has been paid heretofore is the part which it plays in the metabolism of the fatty acids. The fatty acid esters of cholesterol are almost as generally distributed in the animal body as cholesterol itself. Moreover, it has been shown that during the absorption of cholesterol from the intestine considerable quantities of esters are formed.[1,9] It has been claimed by several investigators that the cholesterol of the blood is increased during the absorption of fat free from cholesterol,[19,20] although this finding has not been confirmed by recent work.[23] Further evidence of the participation of cholesterol in fat metabolism is furnished by recent investigations[21,22] on the partition of the lipoids in normal and pathological blood, in which it was shown that cholesterol bears a strikingly constant relation to the other blood lipoids, a relation which was maintained even in a severe diabetic lipemia.[22]

It is desirable in the study of most of these functions and particularly of the part which cholesterol takes in fat metabolism to know the extent to which it is combined with the fatty acids, whether there is normally a balance between the free and bound cholesterol, and whether this balance is maintained in abnormal conditions. A few results have been reported to show that there is in most animals a definite balance between free and bound cholesterol in normal blood[1,23,24] and that this balance may be disturbed in certain abnormal conditions.[23] In order to confirm and extend these findings for human blood the following study was undertaken. The blood samples examined were from a number of normal men and women and from hospital patients of both sexes, the lipoid values of

whose blood have already been reported.[21, 22] In addition there were a number of others obtained mainly from the wards of the Massachusetts General Hospital through the kindness of Dr. W. Denis.

The methods for cholesterol and cholesterol esters have been reported recently.[25, 26] They consist in determinations of these substances in extracts of the blood before and after precipitation of the free cholesterol with digitonin. For total cholesterol 10 cc. of the extract were ordinarily used and 20 cc. for the cholesterol esters. The results are given in the tables.

TABLE I.

Cholesterol and Cholesterol Esters in Human Blood. Mg. per 100 Cc.

Normal.				Pathological.			
Men.	Total.	As ester.		Men.	Total.	As ester.	
	mg.	*mg.*	*per cent*		*mg.*	*mg.*	*per cent*
	193	69	35.7	Diabetes.	332	155	46.6
	201	65	32.3		316	156	49.3
	180	59	32.8		197	71	36.0
	180	62	34.4		299	135	45.1
	178	60	33.7		344	150	43.6
				Pernicious anemia.	156	62	39.7
Average....	......	......	33.8	Hemophilia.	156	52	33.3
Women.				Women.			
	195	60	30.8				
	177	60	33.9	Diabetes.	382	203	53.1
	197	61	30.9	"	352	199	56.5
	184	60	32.6	Pernicious anemia.	166	63	38.0
	208	87	41.8	" "	156	62	39.7
	205	61	29.7	Carcinoma.	212	75	35.3
				"	180	42	23.3
Average....	......	......	33.3	Uremia.	212	72	34.0
				Hyperthyroidism.	238	81	32.0
				Typhoid.	185	82	44.3
				Goiter.	247	83	33.6
				Asthma.	172	63	36.6
				Pregnancy.	291	160	54.9
				"	181	85	47.0
				"	250	150	60.0

TABLE II.

Cholesterol and Cholesterol Esters in Human Blood Plasma. Mg. per 100 Cc.

Normal.

Men.	Total.	As ester.	
	mg.	*mg.*	*per cent*
	202	120	59.4
	207	116	56.1
	208	127	61.0
	200	117	58.5
	245	120	49.0
	180	117	65.0
	291	164	56.3
	190	90	47.4
	192	109	56.7
	210	117	55.7
	198	112	56.5
	196	117	59.6
	208	128	61.5
	203	123	60.6
	220	150	68.2
	195	117	60.0
	221	101	45.7
Average....	......	......	57.5
Women.			
	227	126	55.5
	210	133	63.3
	247	137	55.4
	217	152	70.0
	195	121	62.0
	140	92	65.7
	163	83	50.9
	175	102	58.2
	152	86	56.5
Average....	......	......	59.7

Pathological.

Men.	Total.	As ester.	
	mg.	*mg.*	*per cent*
Diabetes.	250	102	40.8
	282	170	60.3
	396	208	52.5
	454	272	59.9
	470	253	53.8
	476	223	46.8
	508	218	42.9
	538	250	46.4
	577	240	41.6
	915	505	55.1
	1,060	594	56.0
Pernicious anemia.	150	100	66.6
	149	66	44.3
Nephritis.	161	58	36.0
	170	86	50.0
	165	74	44.8
	195	115	58.9
Carcinoma.	178	64	36.0
Hemophilia.	158	103	65.2
Addison's disease.	252	130	51.5
Women.			
Diabetes.	182	116	63.7
	272	166	61.0
	275	180	65.5
	356	211	59.2
	455	250	54.9
	559	358	64.0
	593	356	60.0
Pernicious anemia.	149	66	44.2
	170	106	62.0
	170	110	64.7
	173	110	63.6
	150	88	58.6
	160	125	78.0
	140	90	64.3
Carcinoma.	181	51	28.1
	187	78	41.5
	233	82	35.2
	240	86	3[illegible].0
Hyperthyroidism.	199	62	31.1
Anemia with leukemia.	210	106	50.6
Uremia.	255	78	30.[illegible]

RESULTS AND DISCUSSION.

In normal whole blood the average percentage of combined cholesterol was found to be 33.8 for men, with variations from 32 to 36; in women 33.3 with variations from 30 to 42 per cent.

In normal plasma the average percentage of cholesterol combined as ester in men was found to be 57.5 with variations from 46 to 68; for women, average 59.7 with variations from 51 to 70 per cent. These values agree very well with those reported in the literature.[23, 24] The variations from the average, generally not more than 15 per cent, are the same as those found for other blood lipoids and indicate that these two substances are subject to the same sort of regulation.

In pathological conditions the relation between free and bound cholesterol remains normal in most instances. Even in those cases (diabetes) where the total cholesterol is four or five times the normal value the relation holds constant. Low values for cholesterol esters in the plasma were found in most cases of nephritis and in all the cases of carcinoma examined. The values for diabetic plasma, although within normal limits, are in general definitely below the average, in contrast to the corresponding values in whole blood which are distinctly high. In whole blood the only abnormal values for cholesterol esters, aside from diabetes, were found in pregnancy where they were definitely high, as has also been reported by Herrmann and Neumann.[27]

The constancy of the relationship between free and bound cholesterol in normal individuals indicates that with cholesterol esters as with the other lipoids there is an efficient regulation, and the fact that there is so little variation from the normal relationship in abnormal conditions emphasizes what has been found for most other blood constituents—that the metabolic habits of the individual are not easily upset even by severe disease.

The percentage values for free and bound cholesterol in normal whole blood and plasma bear out the assumption generally made that there is little if any bound cholesterol in the corpuscles. However, in certain of the abnormal conditions noted above, as, for example, diabetes, the relatively low values in plasma together with the high values in whole blood indicate that in some cases the corpuscles may contain combined cholesterol. Further investigation is needed to confirm these indications.

The balance between cholesterol and its esters and therefore between cholesterol esters and the other blood lipoids, gives further ground for the assumption previously made that cholesterol takes an active part in fat metabolism. The possibility cannot of course at present be excluded that this part is secondary to the function of the fatty acids in cholesterol metabolism and that the esters constitute a convenient form in which the excess of cholesterol may be carried in the blood or stored in the tissues (cholesterol esters appear to be the main constituents of the deposits of "anisotropic fats"), but the fact that the blood cholesterol may increase to several times normal (diabetes) or be considerably below normal (anemia) without disturbing the balance speaks against this latter assumption. Determinations of the balance between cholesterol and its esters during fat absorption should give valuable information on this question.

SUMMARY.

In normal human blood there was found a constant relation between free cholesterol and cholesterol esters. In whole blood the average percentage of cholesterol in combination as esters was found to be about 33.5 per cent, in plasma 58 per cent of the total cholesterol. The variation from the average in individuals was about 15 per cent of the average. There was no notable difference in these values between men and women.

In pathological conditions the relation between free and bound cholesterol was found normal in all cases except carcinoma and most cases of nephritis, in both of which the percentage combined as ester was low.

In pregnancy the values for cholesterol esters were high.

The percentage values for free and bound cholesterol in whole blood and plasma in almost all the samples examined bear out the claim that there are few if any cholesterol esters in the corpuscles.

The constant relation between free and bound cholesterol gives further support to the assumption that cholesterol takes an active part in fat metabolism.

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A NOTE ON BENEDICT'S METHOD FOR THE ESTIMATION OF TOTAL SULFUR IN URINE.

By MAURICE H. GIVENS.

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(Received for publication, December 6, 1916.)

The determination of the amount of total sulfur, and consequently the "neutral" sulfur content of urine, demands an accurate method, if any reliance is to be placed upon the values for the latter factor. Folin (1), Benedict (2), Ritson (3), Gill and Grindley (4), and others (5) have laid down procedures for the oxidation and estimation of the "neutral sulfur compounds" in urine. Of all of these only Benedict's method seems to have survived the storm of criticism. In principle it depends upon the oxidation of the urine with copper nitrate.

Besides the proof offered by its originator, and the fact that it is included by several authors in their text-books there is additional evidence (6) that Benedict's method is satisfactory. Schmidt (7), in whose hands it gave concordant results, found that it compared favorably with Folin's method. He had some slight difficulty, however, and says: "Any slight loss due to spattering, which may sometimes take place in oxidizing with copper nitrate, can be prevented by covering the evaporating dish with a watch-glass." Such a procedure may prevent any loss by spattering, but does not prevent spattering.

Denis (8) attempted to use Benedict's method but this attempt she says, ". . . . resulted in utter failure, as, owing to the violent decrepitation and spattering produced at the beginning of ignition by the sodium or potassium chlorate employed, mechanical loss invariably resulted in every one of the forty attempts made. No amount of care in heating seemed to lessen the spattering." She offered no figures to substantiate the statements, as Benedict (9) himself notes, and, instead of trying to overcome the spattering, proceeded to modify the original method.

In the course of work involving about 100 estimations of total sulfur by the use of Benedict's method, it was found that a 3-heat electric hot plate could be employed to avoid spattering or crepitation. If the procedure later outlined is followed these difficulties disappear. Consistent results can be obtained, as the few following typical duplicate analyses of 10 cc. samples of urine indicate:

$BaSO_4$ mg.	Difference. mg.
71.7	0.1
71.6	
32.2	0.5
31.7	
18.6	0.3
18.3	
42.6	0.1
42.7	
50.3	0.5
49.8	
48.6	0.1
48.5	
56.4	
56.0	

We have used the method as follow
ered into a new 150 cc. cass
the dish is washed down
The casserole is then plac
out further attention; o
sacrifice some time in w
In about 45 minutes to
be evaporated to dryn
enough to cause the ni
scrole is removed from
sen, and heated to the
minutes to drive off all
dish is cooled, the

HCl, and diluted with water to about 100 cc. The sulfates present are precipitated with 10 cc. of 10 per cent barium chloride from a dropping tube.

After a good many determinations it may be necessary to filter after dissolving in HCl, as the porcelain may begin to chip off from the heat.

The use of the hot plate does away with all watching and care during evaporation. The heat is sufficient for the oxidation. The number of analyses that can be carried out simultaneously is limited only by the size of the hot plate.

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4. Gill, F. W., and Grindley, H. S., *J. Biol. Chem.*, 1909, vi, p. xi; *J. Am. Chem. Soc.*, 1909, xxxi, 52.
5. For a review of older methods see the paper by Ritson (3).
6. Stadtmüller, N., and Rosenbloom, J., *Arch. Int. Med.*, 1913, xii, 276.
7. Schmidt, C. L. A., *J. Biol. Chem.*, 1910–11, viii, 423.
8. Denis, W., *J. Biol. Chem.*, 1910–11, viii, 401.
9. Benedict, *J. Biol. Chem.*, 1910–11, viii, 499.

THE CHEMICAL COMPOSITION OF THE PLACENTA.

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It is well known that no continuity exists between the maternal and fetal circulations and that interchanges of nutritive material and waste products take place by diffusion through the capillary walls and other membranes at the place or places of contact between the uterus and the fetus.

In the discoidal placenta of the human race the maternal and fetal parts are so closely related that it is impossible to separate them quantitatively, and this placenta is consequently less suitable for some experimental purposes. In the multiple placenta of the cow, the fetal parts are readily and completely separated from the maternal parts and for this reason this placenta was chosen for the present work which was carried out for the purpose of determining the constituents of these two portions of the placenta.

It may be in order to give here a short description of the placental mechanism in the uterus of the cow.[1] With the development of the fetus the uterus undergoes important anatomical and physiological modifications. When gestation has commenced the surface of the maternal caruncles, previously smooth, becomes convex, and is covered with reticulate processes which border the crypts and give it a finely cribbled appearance. The largest are found in the body of the uterus and they become smaller as they approach the extremity of the cornua. In shape they resemble mushrooms, and their color is dark yellow. They constitute the maternal portion of the placenta. The villi of the chorion are developed and agglomerated in larger numbers at certain points of its surface to constitute a multiple polycotyledonary or tufted placenta which is composed in this way of sixty to eighty placental or fetal cotyledons. They are bright red, oval, concave patches 0.4 to 0.6 inches thick and correspond to the maternal cotyledons of the uterus into which they are received.

[1] Craig, J. F., Fleming's Veterinary Obstetrics, London, 1912.

Two stages of pregnancy were selected for the collection of samples from cows, the first with fetuses 3 to 4 months old and the second with almost mature fetuses 8 to 9 months old. In both instances twelve pregnant uteri were employed. These were taken at random on different days, from several hundred pregnant uteri, and the final samples, therefore, are good average representatives of the two stages of pregnancy. Twelve cotyledons from each uterus were removed, carefully trimmed, and the fetal and uterine portions separated by hand. This is an easy matter in this case as the two parts pull apart readily. The fetal and maternal placentulas were again trimmed and weighed, finely minced, and dried *in vacuo* at 35–50°C. The desiccated material was coarsely ground and extracted with petroleum ether in a Soxhlet apparatus.

The maternal cotyledons are grayish yellow in color, while the young fetal cotyledons are pale pink and the older ones dark red. The former are considerably larger than the latter but the sizes of both are in direct proportion to the age and weight of the fetus.

The petroleum ether-soluble substances were obtained by extraction of the desiccated materials with this solvent. After extraction, the liquids were filtered and evaporated on a water bath with a current of air and finally dried *in vacuo* at 50°C. to constant weight. During extraction it was noticed that the uterine placenta gave a bright yellow color to the petroleum ether and the fetal placenta a deep orange color, indicating that both placentas contain distinct coloring matters soluble in the usual fat solvents. After evaporation the products maintained their colors. They were all semisolid and had a strong lecithin odor. Total phosphoric acid was determined on these samples and the amounts of phosphatides estimated as lecithin by multiplying the P_2O_5 figures by 11.24.

Table I gives the average weight of the maternal cotyledons and fetal placentulas, the percentage of moisture and petroleum ether-soluble material in the fresh tissue, and the yield of desiccated fat-free material, also the percentage of phosphatides calculated as lecithin in the fresh tissue. On the desiccated fat-free material was determined moisture, total nitrogen, and ash. The mineral constituents of the four samples were also determined.

TABLE I.

	Fresh tissue.						Desiccated fat-free material.											
	Average weight.	Moisture.	Petroleum ether-soluble substances.	P_2O_5 in petroleum ether-soluble substances.	Phosphatides calculated as lecithin in fresh tissue, $P_2O_5 \times 11.24$.	Desiccated fat-free material.	Moisture.	Ash.	Total nitrogen.	Protein ($N \times 6.25$).	Phosphoric acid (PO_4).	Sulfuric acid (SO_4).	Chlorine (Cl).	Potassium (K).	Sodium (Na).	Iron (Fe).	Calcium (Ca).	Magnesium (Mg).
	gm.	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Maternal placentulas (uterine cotyledons) 3 to 4 months old........	13.6			2.10	0.34	15.35	5.55	6.50	13.17	82.30	3.95	1.85	0.17	1.51	0.85	0.014	0.179	0.115
Dry basis..............								6.88	13.95	87.15	4.18	1.96	0.18	1.60	0.90	0.015	0.189	0.122
Original basis..........		83.20	1.45*					1.06	2.14	13.38	0.642	0.310	0.028	0.246	0.138	0.002	0.029	0.019
Maternal placentulas (uterine cotyledons) 7 to 9 months old........	36.6			2.22	0.37	16.52	4.45	6.38	13.41	83.81	3.77	1.79	0.15	1.71	0.71	0.02	0.179	0.127
Dry basis..............								6.68	14.04	87.75	3.96	1.87	0.157	1.79	0.74	0.021	0.187	0.133
Original basis..........		82.00	1.48*					1.10	2.32	14.50	0.654	0.309	0.026	0.296	0.122	0.003	0.031	0.022
Fetal placentulas (fetal cotyledons) 3 to 4 months old............	10.4			2.50	0.19	10.91	7.05	10.25	12.03	75.19	3.92	1.56	2.06	1.87	1.73	0.028	0.457	0.109
Dry basis..............								11.03	12.94	80.89	4.22	1.68	2.22	2.02	1.86	0.030	0.492	0.117
Original basis..........		88.40	0.69†					1.20	1.41	8.83	0.461	0.183	0.242	0.220	0.203	0.003	0.054	0.013
Fetal placentulas (fetal cotyledons) 7 to 9 months old............	27.6			1.51	0.14	14.96	5.05	6.95	13.18	82.37	3.65	1.62	0.49	1.44	0.96	0.063	0.159	0.120
Dry basis..............								7.32	13.89	86.70	3.84	1.71	0.52	1.52	1.01	0.066	0.167	0.126
Original basis..........		84.20	0.84†					1.09	2.08	12.97	0.574	0.256	0.078	0.228	0.151	0.010	0.025	0.019

* Yellow; semisolid; lecithin odor.

† Dark orange; semisolid; lecithin odor.

Total nitrogen determinations were made according to the official Gunning-Kjeldahl method. The phosphoric acid was determined both in the petroleum ether-soluble substance and in the desiccated fat-free material by heating 1 gm. of the substance with 2 cc. of sulfuric acid and several small portions of nitric acid, until oxidation was complete. The clear liquid was diluted with water and the phosphorus pentoxide determined by the official volumetric method.

The sulfur, iron, calcium, and magnesium determinations were made in the usual way after previous fusion of the desiccated fat-free substance with fusion mixture. The sodium, potassium, and chlorine were determined on the ash which was incinerated at a temperature below dull red heat.

The tabulated results show that the maternal cotyledons contain less moisture and more protein and fats, phosphatides, etc., than their corresponding placentulas. The older uterine cotyledons contain more potassium and less sodium than the younger, but otherwise the variations in the mineral constituents are comparatively slight at the two selected stages of pregnancy.

The young fetal placenta contains much more chlorine and considerably more sodium and calcium and less of the other inorganic constituents than the older fetal placenta. It is evident, therefore, that chlorine, sodium, and calcium are of vital importance in early fetal life. Later, during the rapid growth periods, the amounts of these three elements diminish while the quantities of phosphorus, sulfur, potassium, iron, and magnesia increase.

The 3 to 4 months old fetal placentulas, which are of a pale pink color, contain only about one-third the quantity of iron that is present in the older and dark red fetal placentulas. This indicates that the amount of hemoglobin in the fetal blood is small during the earlier stages of pregnancy, but increases in direct proportion to the growth of the fetus.

All four samples gave negative tests for epinephrin and iodine when lean meat was employed for control analyses.

5 gm. portions of the dry powders were macerated at room temperature with 50 cc. of water, and iodine solution was added until a permanent yellow color remained. After standing for some time the uterine liquids developed a pronounced port wine coloration similar to that produced by addition of iodine to glycogen solutions. The color appeared first in the finer particles of the sediment and was gradually yielded to the liquid. This did not occur in the young fetal extract, which remained light yellow. The old fetal maceration was of a dark red color which prevented

observation. This test, therefore, seems to indicate that glycogen is stored in the uterine portion of the placenta during pregnancy but not in the fetal part. No attempts were made to separate or identify the glycogen further.

In an attempt to separate the liquid from the tissue, portions of the finely minced fresh substances, both fetal and uterine, were centrifuged in a large powerful machine for 1½ hours at a speed of 3,200 revolutions per minute. The cells, however, hold the liquid very firmly as it was possible to obtain only a few cc. from 200 gm. portions of the various samples. The liquids were all faintly acid to litmus, opalescent, and light orange-colored from the uterine placentas. The older fetal placenta yielded dark red, and the younger yellowish pink fluids.

SUMMARY.

The constituents of the uterine placenta seem to remain comparatively uniform throughout gestation, while the fetal placenta varies considerably in composition at different stages of pregnancy. Analytical data of the two portions of the placenta are given and the results discussed.

Total nitrogen determinations were made according to the official Gunning-Kjeldahl method. The phosphoric acid was determined both in the petroleum ether-soluble substance and in the desiccated fat-free material by heating 1 gm. of the substance with 2 cc. of sulfuric acid and several small portions of nitric acid, until oxidation was complete. The clear liquid was diluted with water and the phosphorus pentoxide determined by the official volumetric method.

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THE ESTIMATION OF HYDROCYANIC ACID AND THE PROBABLE FORM IN WHICH IT OCCURS IN SORGHUM VULGARE.

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INTRODUCTION.

The biochemistry of cyanogen compounds has received a considerable amount of attention, not only because of their practical importance in such substances as bitter almonds, linseed cake, fodder grasses, etc., but also because of the theoretical interest which attaches to their probable function in plants. From either viewpoint, facts of first importance to be ascertained are the nature of the cyanogenetic compounds, means of estimating them quantitatively, their situation in the plant, and their reaction to mechanical and chemical treatment of the plant. Although the first two points have received more attention than the others, they are still far from being answered satisfactorily. It is the object of the present paper to describe some experiments on *Sorghum vulgare* bearing on these questions.

Concerning the form in which prussic acid occurs in plants, it is generally believed that it exists free in but few cases, being usually combined as glucosides. About eight such glucosides have been isolated and identified. The nature of the cyanide body, however, is still unknown in the great majority of the plants which are known to contain the cyanide group. Only a few cases are on record where the evidence pointed definitely to the existence of uncombined hydrocyanic acid. Treub[1] found it "free or in a very loosely combined state" in *Pangium edule*, a tropical

[1] Treub, M., Sur la localisation, le transport, et le rôle de l'acide cyanhydrique dans le *Pangium edule*, *Ann. Jardin Bot. Buitenzorg*, 1896, xiii, 1.

tree containing as high as 0.3 per cent of the dry weight of the leaves as hydrocyanic acid. Greshoff,[2] with the same plant, came to similar conclusions. Later Peche,[3] in working out his microchemical test for cyanide, found it free or "in a very labile form" in both *Pangium edule* and in *Prunus laurocerasus*. Moore[4] believed that he had free hydrocyanic acid in cassava. The writer, however, questions this interpretation of his results, as will be discussed later.

Concerning the quantitative estimation of cyanogenetic compounds, it has usually been accomplished by the estimation of the cyanide itself, since it is impossible to extract and crystallize out the glucosides quantitatively. And since in most instances the hydrocyanic acid itself, and not the glucoside, is the critical element under observation, the investigator is content to determine the total cyanide present and disregard any other bodies which may be combined with it. A glucoside linkage is usually assumed, and the plant tissues treated with a view to hydrolyzing this glucoside and setting free the hydrocyanic acid. This is done (1) by autolysis (maceration with water), (2) by added enzyme (emulsin), or (3) by boiling with acids. Autolysis has been used frequently; in fact, the first discovery of hydrocyanic acid in the plant world by Böhm in 1803[5] was in water which had been in contact with crushed bitter almonds. The writer used it in his first studies on the dhurrin in sorghum.[6] Autolysis depends upon the coincident existence of glucoside and glucosidase in the same tissues, but not in contact with each other until the tissues are crushed. Since in a given tissue the enzyme must be perfectly specific for its accompanying glucoside, this method of hydrolysis, where applicable, is very satisfactory. Emulsin seems to act on all cyanogenetic glucosides; and, although its action is less rapid

[2] Greshoff, M., Distribution of prussic acid in the vegetable kingdom, *Report Brit. Assn., York*, 1906, 138.

[3] Peche, K., Mikrochemisher Nachweis der Cyanwasserstoffsäure in Prunus laurocerasus L., *Sitzungsb. k. Akad. Wien*, 1912, cxxi, 33.

[4] Moore, C. C., Cassava: its content of hydrocyanic acid and starch and other properties, *U. S. Dept. Agric., Bureau of Chemistry, Bull. 106*, 1907, 1.

[5] Böhm, *Neues allgemein. J. Chem.*, 1803 (original not consulted).

[6] Willaman, J. J., and West, R. M., Notes on the hydrocyanic-acid content of sorghum, *J. Agric. Research*, 1915, iv, 179.

than that of the autolyzing enzymes, it is nevertheless a highly effective agent in bringing about the hydrolysis of various glucosides. Acid hydrolysis in most cases gives the same final products as does enzyme hydrolysis, but probably does not go to completion or follows a different course. Caldwell and Courtauld,[7] using a 10 per cent solution of amygdalin and normal hydrochloric acid, found only 85 per cent hydrolysis in 200 hours at 60°C., and 92 per cent in 16 hours at 80°C. Walker and Krieble[8] obtained slow and incomplete hydrolysis with hydrochloric and sulfuric, and none with oxalic or trichloroacetic acid. Recently several investigators[9, 10, 11] have used 5 per cent sulfuric or 5 per cent tartaric acid. They judged the completeness of their hydrolysis only by the failure of further hydrocyanic acid to distill over. That this may be a false assumption, at least in the case of certain plants, will be brought out in the following experiments. The writer and West, in the 1915 work on sorghum at the Minnesota Experiment Station,[12] following the directions of Viehoever and Johns, ground the leaves in a food chopper and then distilled from 5 per cent tartaric acid. No further cyanide was obtained after an hour's distillation. That cyanide obtained in this way is not the product of acid hydrolysis, but of enzyme hydrolysis taking place previous to the addition of the acid, is in part the object of the present paper to show.

The success of any method for the determination of prussic acid depends not only on the complete hydrolysis of the glucoside, but also on the complete removal of the cyanide from the hydrolysate. A thorough discussion of this latter phase of the question was given by Alsberg and Black.[10] In their experiments with

[7] Caldwell, R. J., and Courtauld, S. L., The hydrolysis of amygdalin by acids, *J. Chem. Soc.*, 1907, xci, 666.

[8] Walker, J. W., and Krieble, V. K., The hydrolysis of amygdalin by acids, *J. Chem. Soc.*, 1909, xcv, 1369.

[9] Viehoever, A., and Johns, C. O., On the determination of small quantities of hydrocyanic acid, *J. Am. Chem. Soc.*, 1914, xxxvii, 601.

[10] Alsberg, C. L., and Black, O. F., The separation of autogenous and added hydrocyanic acid from certain plant tissues and its disappearance during maceration, *J. Biol. Chem.*, 1916, xxv, 133.

[11] Viehoever, A., Johns, C. O., and Alsberg, C. L., Cyanogenesis in plants. Studies on *Tridens flavus* (tall red top), *J. Biol. Chem.*, 1916, xxv, 141.

[12] Willaman and West, Effect of climatic factors on the hydrocyanic acid content of sorghum, *J. Agric. Research*, 1916, vi, 261.

several cyanogenetic plants they noticed a loss of hydrocyanic acid when the macerated tissue was allowed to stand some hours before distilling from acid. Also, added cyanide was lost in the same way. A non-cyanogenetic plant, *Sambucus canadensis*, did not have this power. They proved that the phenomenon was not due to enzymes, and suggested that there was a chemical reaction between the hydrocyanic acid and some other substances in the tissues, probably aldehydes. In another paper by Viehoever, Johns, and Alsberg,[11] retention of hydrocyanic acid on maceration was reported in *Tridens flavus;* maceration in presence of tartaric acid prevented this retention. Henry and Auld[13] devised a scheme which was designed to prevent retention of cyanide by the macerated tissue. They extracted the material with alcohol, evaporated the alcohol, then took up with water, and distilled with 2 per cent HCl. The cyanogenetic compounds were no doubt removed in this way, but whether they were all hydrolyzed by the acid is doubtful.

It is evident that there is considerable cause for questioning our knowledge of the actual amount of hydrocyanic acid present in various plants and the conditions in which it exists there. Most workers in this field have not taken into account (1) the possible incomplete liberation of hydrocyanic acid from its compounds, (2) the possible incomplete removal of the acid by distillation, and (3) the existence of various states of combination between the hydrocyanic acid and other constituents of the plants. The following experiments deal with these three points in the cyanogenesis of sorghum.

EXPERIMENTAL.

Material and Method.

The sorghum used in these experiments was of the Early Amber variety grown on the plots of the Minnesota Agricultural Experiment Station. Most of the samples were taken between the stages of full bloom and maturity, when the plants were 6 feet high or over. The leaves only were used.

The distilled hydrocyanic acid was collected in an excess of sodium hydroxide, and determined colorimetrically by the Prussian blue method of Viehoever and Johns.[9]

[13] Henry, T. A., and Auld, S. J. M., The occurrence of cyanogenetic glucosides in feedingstuffs, *J. Soc. Chem. Ind.*, 1908, xxvii, 428.

The procedure with some samples furnished illustrations for more than one factor under observation. Hence in the tables considerable duplication of laboratory numbers will be noticed. Instead of describing and discussing the procedure followed for each sample of leaves in chronological order and noting the various points observed, each factor (as acid hydrolysis, autolysis, etc.) will be taken up by itself, and in the tables reference will be made to the experiments from which the data were obtained.

Comparison of Acid and Enzyme Hydrolysis.

In order to determine the efficiency of various acids as hydrolyzing agents for cyanogenetic glucosides, the trials described in Table I were carried out, using amygdalin as the glucoside. Hydrolysis by emulsin was also performed for comparison.

TABLE I.

No.	Method of hydrolysis.	HCN		
		Present.	Recovered.	
		mg.	*mg.*	*per cent*
53	34.4 mg. amygdalin distilled $1\frac{1}{4}$ hrs. with 5 per cent tartaric	2.00	0.00	0.0
54	34.4 " " distilled $1\frac{1}{4}$ hrs. with 5 per cent H_2SO_4	2.00	0.00	0.0
55	200 " " in 40 cc. water, 100 mg. emulsin, 24 hrs. at 45°C.	10.56	10.40	98.5
56a	200 " " distilled $2\frac{1}{4}$ hrs., 200 cc. 5 per cent H_2SO_4	10.56	0.40	3.8
56b	200 " " distilled $2\frac{1}{4}$ hrs., 200 cc. 5 per cent HNO_3	10.56	0.00	0.0
56c	200 " " distilled $2\frac{1}{4}$ hrs., 200 cc. 5 per cent tartaric	10.56	0.00	0.0
57	200 " " boiled 24 hrs. with 200 cc. 5 per cent H_2SO_4*	10.56	0.70	6.7
58a	50 " " 10 mg. emulsin, 47°C. 4 hrs.	2.64	2.20	83.3
58b	50 " " 5 mg. emulsin, 47°C. 4 hrs.	2.64	1.40	53.0
73	200 " " 200 cc. 2 per cent HCl, 72 hrs. boiling	10.56	2.80	26.5

* The material was boiled under a reflux connected with bulbs of NaOH. After the cyanide had been determined, the ammonia was distilled off; 1.0 mg. of N as NH_3 was obtained, corresponding to 1.9 mg. of HCN.

The results show that no acid is at all efficient for the hydrolysis of amygdalin. As evidenced in No. 57, considerable of the hydrocyanic acid in the glucoside is set free as ammonia by strong acids, according to the well known mandelonitrite decomposition. Emulsin proved a very efficient hydrolyzing agent. How far we are safe in assuming that what is true for amygdalin is also true for dhurrin, we cannot say at present. However, the succeeding experiments will show that dhurrin is probably not hydrolyzed by acids to any greater extent than is amygdalin.

Effect of Autolysis.

In Table II are collected the data obtained in the various experiments dealing with the autolysis of the macerated sorghum leaves. The procedure in general was to grind the leaves in a food chopper, and then put them into 300 cc. of water for autolysis, or into 300 cc. of 5 per cent tartaric acid for distillation. It was not expected that the acid would hydrolyze any dhurrin; it merely furnished an acid medium from which to distill the hydrocyanic acid present in the tissues from any source whatsoever, and prevented enzyme action. In order to preclude entirely any chance for enzyme action on the dhurrin, in some cases the leaves were moistened with the tartaric acid before grinding, and then were ground directly into more of the acid. In the tables this method is called "grinding with tartaric," and is used as a check determination in most cases to judge the effect of autolysis.

In Nos. 60 and 62 there is a marked increase in hydrocyanic acid after hydrolysis; there is no increase in Nos. 70 and 65, where no tartaric acid was added before distilling. This is the reverse of what was suggested by Viehoever, Johns, and Alsberg.[14] They did not offer an explanation of why the addition of acid after the period of autolysis should affect the retention of cyanide. In Nos. 87 and 84, the check was ground with tartaric; the other sample had a chance to autolyze from the moment the tissues were ruptured till the macerated mass was heated to the killing temperature of the enzyme, probably 10 minutes. This was evidently sufficient time for considerable hydrolysis to take place,

[14] Viehoever, Johns, and Alsberg, Cyanogenesis in plants. Studies on *Tridens flavus* (tall red top), *J. Biol. Chem.*, 1916, xxv, 142.

but insufficient for much "retaining" action of the tissues on the hydrocyanic acid. In comparison to this, Nos. 87 and 85 show complete retention in 16 hours' autolysis. Two examples of 4 hour autolysis are given in Nos. 88a and 88b, and Nos. 91a and 91b. There is a marked increase in both cases.

It is apparent from these data that the hydrolysis of dhurrin in sorghum leaves by the action of the contained glucosidase takes place rapidly; it is probably completed in a few hours, perhaps in a few minutes. Auld[16] found that when linseed cake was autolyzed in water at 37°C., most of the hydrocyanic acid was liberated in 15 minutes, and all of it in 6 hours.

TABLE II.

The Effect of Autolysis of Sorghum Leaves for Various Lengths of Time, on the Yield of Hydrocyanic Acid.

Nos.	Conditions of autolysis.	Method of determining HCN.	HCN per 100 gm. of dry matter. Before autolysis.	HCN per 100 gm. of dry matter. After autolysis.
			mg.	*mg.*
60 and 62	24 hrs., 47°, 300 cc.	Distilled from 5 per cent tartaric.	7.12	12.25
70 " 65	24 " 47°, 300 "	Distilled from water.	12.40	12.40
87 " 84	10 min., previous to reaching the boiling point.	" " "	1.74	10.45
87 " 85	16 hrs., 47°, 300 cc.	" " "	1.74	0.00
88a " 88b	4 " 23°, 300 "	Check ground with 5 per cent tartaric; autolyzed sample distilled from water.	0.00	5.51
91a " 91b	4 " 23°, 300 "		0.00	7.16

In Moore's investigation of cassava[4] he ground the tissues to a pulp, placed it in a retort, and slowly distilled for 2 hours. Most of the cyanide passed over in the early stages. He says: "From this fact and the nature of the results obtained there is no reason to believe that the figures given represent other than the HCN existing in the free state." He got no increase in cyanide by the

[15] Auld, S. J. M., Formation of prussic acid from linseed cake and other feedingstuffs, *J. Southeast Agric. College Wye*, 1911, no. 20, 289.

use of sulfuric acid. Now, if autolysis in cassava proceeds at anything like the rate it does in sorghum, there was time in Moore's process for considerable liberation of cyanide before the mass of tissue reached a temperature fatal to the enzymes. There may be free hydrocyanic acid in cassava; but it is the writer's conviction that the above process gives considerably more than the non-glucosidic hydrocyanic acid.

In the same way, when Peche[3] concluded that he had free hydrocyanic acid when a section of the tissue gave a reduction of mercurous nitrate, it is possible that the act of sectioning brought glucoside and enzyme in contact for a time sufficient to set free enough hydrocyanic acid to give a test. By the time these facts on sorghum were ascertained, material was not available for making a more thorough study of this rate of autolysis. The writer believes, however, that for each plant species an optimum time for autolysis can be found during which a maximum yield of hydrocyanic acid can be obtained. This may not represent all the cyanide present; it probably is the difference between the total amount set free and the amount retained by the macerated tissue. What may be the nature of this retention is not known; it is suggested that, if aldehydes are responsible, some substance may be found which, added to the macerated tissue, will combine with the aldehydes and prevent their action on the cyanide. De Jong,[16] in working with the decomposition of gynocardin in the leaves of *Pangium edule*, found that a diketone and HCN were produced, which combined in part, thus holding back some of the prussic acid from distillation.

It is also evident from Table II that grinding with tartaric acid completely inhibits enzyme action, as in two cases, Nos. 88a and 91a, no cyanide was obtained in this way, whereas by normal hydrolysis from 5 to 7 mg. were obtained. In No. 87, where cyanide was obtained even with the acid treatment, this cyanide no doubt existed in some non-glucosidic form. This will be discussed under another heading.

[16] De Jong, A. W. K., La décomposition de la gynocardine par l'enzyme des feuilles de pangium edule, *Rec. trav. chim. Pays-Bas*, 1911, xxx, 220.

Effect of Vacuum Distillation on the Yield of Cyanide.

In order to ascertain whether distillation under reduced pressure, and hence lower temperature, would decrease the retention of cyanide, the experiments in Table III were carried out. After removing the cyanide under reduced pressure, redistillation was found to be necessary because foaming had discolored the distillate. It is unlikely that this redistillation had any effect on the cyanide. In two of the cases a slight increase in hydrocyanic acid in favor of vacuum distillation is seen, but it is inappreciable.

TABLE III.

The Effect of Distilling Macerated Sample 1½ Hrs. at 50–55°, 15 Mm. Pressure, Then Redistilling at Ordinary Pressure.

Nos.	Description of samples.	HCN obtained.	
		By ordinary distillation.	By vacuum distillation.
		mg.	*mg.*
72a and 72b	Ground with 5 per cent tartaric.	0.40	0.40
76a " 76b	" " 5 " " "	0.00	0.20
82 " 83	" " 5 " " " and 1.0 mg. HCN (as KCN) added.	0.60	0.80

Form of the Occurrence of Hydrocyanic Acid in Sorghum.

In the discussion of the data in Table II, it was pointed out that grinding in the presence of tartaric acid completely prevented enzyme action on glucoside. Nevertheless, cyanide was obtained in this way in some cases. Assuming that the acid cannot appreciably affect dhurrin, this cyanide must have existed in the plant in some non-glucosidic form. The only reference the writer could find on the nature of the hydrocyanic acid compounds of sorghum was one by Dunstan and Henry,[17] who, in discussing their newly found sorghum glucoside dhurrin, said that the hydrocyanic acid apparently does not occur in the free state, but only as the glucoside. They did not state their reasons for this conclusion. In Table IV is brought together the evidence which

[17] Dunstan, W. R., and Henry, T. A., Cyanogenesis in plants. II. The great millet, *Sorghum vulgare*, *Chem. News*, 1902, lxxxv, 301.

points towards the existence in Sorghum vulgare of non-glucosidic hydrocyanic acid. It all hinges upon two assumptions: first, that the method of grinding with 5 per cent tartaric acid prevents any

TABLE IV.

The Occurrence of Glucosidic and Non-Glucosidic Hydrocyanic Acid in Sorghum.

Nos.	HCN per 100 gm. of dry weight of leaves.		
60, 61, 62	I. Leaves ground, then distilled from 5 per cent tartaric, allowing about 5 min. autolysis. 7.12 mg.	II. Leaves ground and distilled from water, allowing about 8 min. autolysis. 7.12 mg.	III. Autolyzed 24 hrs. at 45°, then distilled from tartaric. 12.25 mg.
69, 70, 65	I. Ground with tartaric, all enzyme action prohibited. 5.13 mg.	II. Ground and distilled from water, about 8 min. autolysis. 12.40 mg.	III. Autolyzed 24 hrs. at 45°. 12.40 mg.
76a, 87, 84	I. Ground with tartaric, all enzyme action prohibited. 0.00 mg.	II. Same sample as I, but frosted, ground with tartaric, all enzyme action prohibited. 1.74 mg.	III. Same sample as II, ground and distilled from water, about 8 min. autolysis. 10.45 mg.
89a1 and 82a2	I. Ground with tartaric, all enzyme action prohibited. 0.00 mg.	II. Ground and autolyzed 4 hrs. at 23°. 4.50 mg.	
90a1 and 90a2	I. Ground with tartaric, all enzyme action prohibited. 4.50 mg.	II. Ground and autolyzed 4 hrs. at 23°. 11.25 mg.	

enzyme decomposition of glucoside; and second, that the acid itself does not accomplish any hydrolysis even during boiling. The first is based upon the fact that a given sample of sorghum

leaves will yield cyanide after autolysis, but will not yield any if ground in presence of tartaric acid; and that still other samples give some cyanide when ground with the acid, but will give more if not ground with it. The second assumption is based on the following facts: (1) Amygdalin cannot be hydrolyzed with 5 per cent tartaric acid (see Table I) and amygdalin and dhurrin are very similar in their composition and properties. (2) Samples of leaves which yield hydrocyanic acid by autolysis, yield none on boiling with tartaric acid. Therefore it is believed that sorghum does not contain all of its hydrocyanic acid in the form of dhurrin; that at times part or all of it may exist in some more unstable compound which can be decomposed by boiling 5 per cent tartaric acid. Whether the prussic acid actually exists free in certain of the cells, as found by Treub in *Pangium edule*, or even as the salt of metals, the data at present do not show. Peche[3] suggests that in *Prunus laurocerasus* it may be linked with a ketone. Treub,[1] in demonstrating the presence of free hydrocyanic acid in *Pangium edule*, treated the leaves with boiling alcohol to prevent enzyme action. De Jong[18] modified this by using large volumes of absolute alcohol at −10°C.

This discovery may explain the apparent discrepancy between Auld's[16, 19] results from the study of the possibility of cyanide poisoning in stock, and what actually happens in practice. He concluded, after a study of the conditions favorable for the liberation of cyanide in feedingstuffs by enzymes, that such hydrolysis is unlikely except under unusual circumstances, since it is prevented by the reaction of the alimentary pouches, and by the presence of large amounts of fiber, salt, glucose, etc. Cyanide poisoning, however, is very common in districts growing sorghum, and it is probable that it is caused in large part by the non-glucosidic hydrocyanic acid.

The demonstration of the inefficiency of tartaric acid as a hydrolyzing agent for dhurrin, of the rapidity of autolysis on grinding the tissues, and of the existence of non-glucosidic hydrocyanic acid in sorghum, necessitates a revision of the conclusions arrived

[18] De Jong, L'acide cyanhydrique des feuilles du *Pangium edule*, *Ann. Jardin Bot. Buitenzorg*, 1908, xxii, 1.

[19] Auld, Cyanogenesis under digestive conditions. *J. Agric. Sc.*, 1913, v, 409.

at by Willaman and West[12] in regard to the effect of climatic factors on the prussic acid content of sorghum. The method employed there throughout the season was to grind the tissues, and then put them into 5 per cent tartaric acid. From the evidence given above, this allowed from 4 to 7 minutes for autolysis; a time sufficient for considerable, if not for complete, hydrolysis. If it was complete, or if the degree of hydrolysis was the same in all cases, the comparative value of the data would still obtain. If there was considerable variation in the relative amount of the total cyanide in the non-glucosidic condition, there would also be a corresponding variation in the amount of hydrolysis of the glucoside, and the data would carry but little significance. Which of these two cases is correct remains for future work to determine.

It would be of interest to study the form of occurrence of the prussic acid in the hosts of cyanogenetic plants known to science. No doubt more exact information would be obtained as to the nature of this non-glucosidic union of cyanide, as well as to the variation in the proportion of glucosidic to non-glucosidic cyanide present under various conditions.

SUMMARY.

1. The methods in use for the determination of the hydrocyanic acid content of plant tissues are of questionable accuracy, because of the difficulty in getting complete hydrolysis of the glucosides by means of acids, and because of the retention of the cyanide from distillation by the tissues involved.

2. Hydrolysis of the dhurrin in sorghum is best accomplished by means of the glucosidase found in the same tissues (autolysis). It takes place very rapidly at 45°C.

3. Retention of hydrocyanic acid by the tissues during distillation cannot be prevented by the presence of tartaric acid, nor can it appreciably be lessened by distilling under reduced pressure.

4. *Sorghum vulgare* contains hydrocyanic acid in two forms; a glucosidic, as dhurrin, and a non-glucosidic, the nature of whic is as yet unknown. It is the latter portion of the cyanide of th plant which is probably responsible for the poisoning of stock.

5. The non-glucosidic cyanide can be distinguished from th glucosidic by grinding the leaves in the presence of 5 per cen tartaric acid to prevent any enzyme action, and then distilling.

THE EFFECT OF ANESTHETICS AND OF FROSTING ON THE CYANOGENETIC COMPOUNDS OF SORGHUM VULGARE.

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INTRODUCTION.

Some interesting phenomena have been observed arising from the effect of anesthesia on living plants. The forced premature flowering of certain plants by means of ether is a commercial practice,[1] especially applicable to plants in a dormant condition, as bulbs and twigs in the late winter. It is believed, in general, that the entrance of the anesthetic increases the permeability of the tissues, with a consequent more rapid absorption of water. Some anesthesia phenomena are thus explained by the bringing together of substrates and enzymes.

The most carefully worked out explanation of the effect of anesthetics is contained in the work of the Armstrongs.[2, 3] The material for their study was the leaves of the cherry laurel, *Prunus laurocerasus*, containing the cyanogenetic glucoside prulaurasin. As an index of the effect of anesthetics they used Mirande's[4] test for the liberation of hydrocyanic acid from plant tissue. The test consists in immersing about two-thirds of the

[1] Duggar, B. M., Plant Physiology, New York, 1911, 335.

[2] Armstrong, H. E., and Armstrong, E. F., The origin of osmotic effects. III. The function of hormones in stimulating enzymic change in relation to narcosis and the phenomena of degenerative and regenerative change in living structures, *Proc. Roy. Soc., Series B*, 1909–10, lxxxii, 588.

[3] Armstrong and Armstrong, The function of hormones in regulating metabolism, *Ann. Bot.*, 1911, xxv, 507.

[4] Mirande, M., Influence exercée par certaines vapeurs sur la cyanogenèse végétale. Procédé rapide pour la recherche des plantes à acide cyanhydrique, *Compt. rend. Acad.*, 1909, cxlix, 140.

tissue, as a leaf, for example, in water containing the anesthetic. The latter enters the leaf and brings about the hydrolysis of the contained cyanogenetic glucoside and thus the liberation of hydrocyanic acid, which is transpired from the unimmersed portions of the leaf and produces a color change in moist sodium picrate paper suspended in the tube over the leaf. The time required for the appearance of the color change is a rough index of the intensity and rapidity of the action of the anesthetic. The Armstrongs employed a large number of substances in their attempt to explain the mechanism of the reaction. They found that the substances with the least attraction for water, or the anhydrophyllic, exerted the most marked effect, while the hydrophyllic had the least. Thus chloroform, carbon bisulfide, ether, and ammonia were strongly active; benzene, naphthalene, and thymol were moderately active; while most inorganic salts and weak acids were inactive.

From these results they concluded that anesthetizing substances possess their action by virtue of their lack of attraction for water; that their molecules can pass through the differential septa of plants without becoming involved in the aqueous structure thereof; that "such interposition, however, at once alters the osmotic state by promoting dissociative chemical change in the water in the direction $(H_2O)_x \rightarrow xH_2O$, ... and the equilibrium is disturbed, so that an influx of water from other regions sets in."

The reaction in plants brought about by anesthetics, then, is one of hydrolysis. It is not necessarily the hydrolysis of glucosides, however, for the disturbance in the tissues may bring together any substrate and its enzyme.

One other study similar to that of the Armstrongs should be mentioned. That is the work of Clark[5] on wild indigo. Anesthetics in this case cause the hydrolysis of a glucoside into an aromatic compound, which then is oxidized by an oxidase to a black pigment, causing the whole leaf to turn black. He found acetone and then the lower esters to be the most active in bringing about this effect.

[5] Clark, E. D., Note on the blackening of the leaves of the wild indigo (*Baptisia tinctoria*) and the isolation of a new phenol, baptisol, *J. Biol. Chem.*, 1915, xxi, 645.

In the investigations on cyanogenesis in the common sorghum made by the writer[6] in 1915, chloroform and alcoholic sodium hydroxide were used as a preservative in shipping the plants in cans from various field plots to the laboratory for analysis. As a control measure, samples from the home plots were analyzed before and after preservation, at all stages of growth, to detect any unwonted effect of the preservation in the hydrocyanic acid content. As shown in Table I in the report of those experiments, no effect was noticed through those stages of growth that were involved in the experiments. As the plants become older, however, it became increasingly evident that more cyanide was obtainable after several days' preservation in chloroform than before. A number of experiments were tried, varying different factors, and involving plants of different ages. The plant parts were cut into 1 inch lengths, and 100 gm. were placed in a 500 cc. tin can with a closely fitting cover. The preservative was added and the material allowed to stand at room temperature the specified length of time. The method of analysis was to grind the tissue in a food chopper, add immediately to 5 per cent tartaric acid, and distill slowly into a little sodium hydrate solution until a volume of 100 cc. was obtained. The hydrocyanic acid was estimated colorimetrically by the method of Viehoever and Johns.[7] As was discussed in a previous paper,[8] this tartaric acid distillation does not liberate all the hydrocyanic acid in the tissues; but since all the samples in these experiments received almost identical treatment, the results are comparable one with the other.

Some of the data obtained concerning the effect of the anesthetics are reproduced in Table I. Chloroform, alcohol, and ether, when acting on the older plants, apparently cause an increased yield of prussic acid; this effect is very marked in some cases. The presence of sodium hydrate in the alcohol decreases

[6] Willaman, J. J., and West, R. M., Effect of climatic factors on the hydrocyanic acid content of sorghum, *J. Agric. Research*, 1916, vi, 261.

[7] Viehoever, A., and Johns, C. O., On the determination of small quantities of hydrocyanic acid, *J. Am. Chem. Soc.*, 1915, xxxvii, 601.

[8] Willaman, J. J., The estimation of hydrocyanic acid and the probable form in which it occurs in *Sorghum vulgare*, *J. Biol. C*[illegible]
25.

TABLE I.

The Effect of Various Anesthetic Preservatives on the Hydrocyanic Acid Content of Sorghum.

No.	Method of preservation.	Portion of plant used.	Height of plant.	HCN of dry matter. Without preservation.	HCN of dry matter. With preservation.
			ft.	*per cent*	*per cent*
P 16	20 cc. alcoholic NaOH + 4 cc. $CHCl_3$, 4 days.	Whole plant.	2.4	0.019	0.020
P 18	20 " " " 4 " " 4 "	Whole plant.	2.4	0.026	0.029
P 27	20 " " " 4 " " 8 "	Whole plant.	3.3	0.009	0.009
P 29	20 " " " 4 " " 8 "	Whole plant.	3.3	0.016	0.019
P 32	30 " " " 4 " " 3 "	Leaves.	4.5	0.004	0.014
P 34	30 " " " 4 " " 3 "	"	4.5	0.007	0.031
P 35a	30 " " " 4 " " 3 "	"	5.7	0.004	0.020
P 35b	30 " " " 4 " " 6 "	"	5.7	0.004	0.030
P 21	20 " alcohol, 4 days....................	"	6.5	0.001	0.022
P 22	20 " " + 2 cc. $CHCl_3$, 4 days......	"	6.5	0.001	0.017
P 23	20 " " + 2 " ether, 4 "	"	6.5	0.001	0.017
P 24	20 " 2 per cent alcoholic NaOH..........	"	6.5	0.001	0.009
P 38	No preservative, 2.5 days................	"	6.8	Trace	Trace
P 39	30 cc. alcohol, 2.5 days.................	"	6.8	"	0.012
P 43	30 " " + 2 cc. $CHCl_3$, ½ hr........	"	6.8	"	0.001
P 44	30 " " 2 " " 2.5 days...	"	6.8	"	0.004
P 46	20 cc. alcoholic NaOH + 2 cc. $CHCl_3$, ½ hr.	"	6.8	0.003	0.010
P 47	20 " " " 2 " " ½ "	"	6.8	0.003	0.010

the effect somewhat, probably because of the hardening of the epidermis and the consequent lesser penetration of the alcohol.

Three possible explanations of this phenomenon are suggested: (1) the disturbed osmotic relations in the tissues bring about increased hydrolysis of the cyanogenetic glucoside dhurrin; (2) the presence of the anesthetics stimulates not only the hydrolytic

activities of the enzymes, but their synthetic activities as well, resulting in this case in an actual production of more dhurrin and more hydrocyanic acid; (3) a combination of both (2) and (1).

EXPERIMENTAL.

In order to obtain some further information on this subject, it was decided to carry on a few more experiments on the 1916 crop of sorghum. The plan was to subject a quantity of leaves to the action of chloroform vapor and then to extract and isolate the dhurrin itself, if possible, comparing the yield with that from the same quantity of untreated leaves. The method followed was that of Dunstan and Henry.[9] The leaves were dried in a current of air at about 45°C., ground to a fine powder, extracted twice with 95 per cent alcohol at the rate of 400 cc. of alcohol for each 100 gm. of leaf powder, the alcohol was evaporated, the residue taken up with water, freed from tannates by lead acetate, made into a paste with animal charcoal, dried, extracted in a Soxhlet for 72 hours with anhydrous ethyl acetate, and the extract slowly evaporated to crystallize out the dhurrin. The attempt to obtain crystalline dhurrin, however, resulted in a failure. In the first extraction dhurrin was present, but it was accompanied by too much coloring matter to be recovered in crystalline form. When it was decolorized by a second treatment with charcoal, crystals again failed to form. At each step of the process, however, an analysis was made for hydrocyanic acid, thus enabling one to follow the comparative cyanide content without obtaining the glucoside itself. In some cases the hydrocyanic acid was obtained by autolysis; in others by hydrolysis with emulsin.

The results of the experiments are given in Table II. It will be seen that in every case there is more hydrocyanic acid in the chloroformed than in the normal leaves, as shown by the analyses at each stage of the process. The analysis at each stage, however, has a different significance. In the first pair of samples, the first column in the table shows the prussic acid obtained by about

[9] Dunstan, W. R., and Henry, T. A., Cyanogenesis in plants. II. The great millet, *Sorghum vulgare*, *Phil. Tr. Roy. Soc., Series A*, 1902, cxcix, 399.

TABLE II.

The Effect of Chloroform Vapors on the Cyanogenetic Compounds of Sorghum.

No.	Treatment.	HCN per 100 gm. of dry weight of leaves.			
		I. Original leaves ground, distilled from tartaric acid.	II. Leaves after treatment, ground and autolyzed 24 hrs. at 45°.	III. Leaves after treatment, dried, ground, and autolyzed 24 hrs. at 45° with emulsin.	IV. Dried and ground leaves extracted with alcohol and hydrolyzed with emulsin.
		mg.	*mg.*	*mg.*	*mg.*
52c	300 gm. of leaves packed in can 3 days at room temperature.	0.60			1.15
52d	300 gm. of leaves packed in can 3 days at room temperature with 9 cc. $CHCl_3$.	0.60			1.40
74	600 gm. of leaves same as 52c.	0.40	0.00	1.60	2.80
75	600 gm. of leaves same as 52d, but 18 cc. $CHCl_3$.	0.40	0.00	2.80	3.60
		I. Original leaves ground and autolyzed 4 hrs. at 23°.	II. Leaves after treatment ground and autolyzed 4 hrs. at 45°.	III. Leaves after treatment dried, ground, extracted with alcohol, and hydrolyzed with emulsin at 47° for 3 hrs.	
89	1,800 gm. of leaves packed in can 4 days.	5.51	4.50	0.26	
90	1,800 gm. of leaves packed in can 4 days with 53 cc. $CHCl_3$.	5.51	11.25	2.00	

8 minutes' autolysis previous to the addition of the tartaric acid. and includes, probably, all the non-glucosidic cyanide and part of the glucosidic, except that retained from distillation by the chemical action of the tissues.[10] The cyanide represented in the fourth column can have arisen only from dhurrin, since the drying of the leaves and the evaporation of the alcohol would have entirely removed any volatile cyanide before the hydrolysis by emul-

[10] See previous paper[8] for a discussion of this.

sin was performed. The difference in the yield of hydrocyanic acid here is a difference brought about by the *increased production of dhurrin in the leaves under the influence of the anesthetic.* The same can be said of the next pair of samples, Nos. 74 and 75. The third column shows an increased amount of dhurrin present in the leaves, as obtained by autolysis of the dried and powdered leaves; the fourth column shows an increased amount of dhurrin as obtained by extraction with alcohol. In the second column the prolonged autolysis caused the retention of all of the cyanide. In Nos. 89 and 90, the cyanide liberated by 4 hours' autolysis (second column) was doubled in the chloroformed plants. The glucosidic cyanide, extractable by alcohol (third column), decreased markedly in both, but much less in the treated than in the normal leaves. This shows *increased hydrolysis of the glucoside under the influence of the anesthetic,* and at the same time an *augmented synthesis of hydrocyanic acid.*

Evidently, then, the third proposition enumerated above holds true here. That is, when sorghum leaves undergo anesthesia by chloroform, both the synthetic and the hydrolytic activities of glucoside enzymes are increased. This in effect amounts to a case of demonstrated enzyme synthesis *in vivo.* It does not prove that the same enzyme is responsible for both the hydrolytic and the synthetic action towards dhurrin; in fact it rather proves the reverse, and substantiates Armstrong's hypothesis[11] that different enzymes are involved in the building up and the breaking down of a given substance. Since it is fair to assume that the chloroform affects primarily the enzymic activities of the tissues, then any increased production of glucoside must be due to synthetic enzyme activity.

In order to demonstrate, if possible, that an enzyme preparation from chloroformed leaves would reveal increased hydrolytic activity, enzyme powders were prepared from the residues of Nos. 89 and 90 (Table II) after extraction of the material with alcohol. The residues, freed from alcohol, were extracted with 2,000 cc. of water for 2 hours, filtered, 800 cc. of the filtrate were concentrated to 80 cc. at 30°C. under reduced pressure, poured into 600 cc. of 95

[11] Armstrong, E. F., Studies on enzyme action. III. The influence of the products of change on the rate of change conditioned by sucroclastic enzymes, *Proc. Roy. Soc.*, 1904, lxxiii, 516.

per cent alcohol, filtered immediately, and the precipitate dried. About 12 gm. of powder were obtained from each lot of material. The activities of the powders towards amygdalin were then determined by allowing 5 gm. of each to act on 0.3 gm. of amygdalin in 200 cc. of water at 47°C. for 2 hours. No. 89, from leaves not chloroformed, liberated 0.20 mg. of HCN; No. 90, from chloroformed leaves, liberated 5.00 mg. of HCN. Thus the chloroformed leaves yielded an enzyme powder about twenty-five times as active towards amygdalin as the powder from the same quantity of untreated leaves.

TABLE III.

The Effect of Frost on the Cyanogenetic Compounds of Sorghum.

Nos.	HCN per 100 gm. of dry matter			
	Before frosting.		After frosting.	
	Non-glucosidic HCN.*	*Glucosidic* HCN.†	*Non-glucosidic* HCN.	*Glucosidic* HCN.
	mg.	*mg.*	*mg.*	*mg.*
76a and 87	0.00		1.74	
88 " 91	0.00	5.51	0.00	7.16

* Determined by grinding leaves with 5 per cent tartaric acid, thus preventing any enzyme action.

† Determined by grinding, then autolyzing 4 hrs. at 23°.

Sorghum has the reputation of being especially poisonous after it has been frosted. The question arose as to whether this increased toxicity was not due to a set of circumstances similar to those associated with anesthesia. To prove the point, a sample of frosted sorghum leaves (No. 87, Table III) was obtained on September 18, and analyzed for non-glucosidic hydrocyanic acid. Leaves from these same plants (No. 76a) on September 13, before being frosted, had already been analyzed. The results are given in Table III. In this case the frosting of the leaves had caused *profound hydrolytic changes.* Next, a sample of unfrosted leaves was obtained, and exposed in a refrigerating plant for 1¼ hours to a temperature of -7°C. After removal from here they were allowed to stand at 20°C. for 20 hours, and then analyzed, with the results shown in the table (Nos. 88 and 91). In this case no non-glucosidic hydrocyanic acid was obtained either before or af-

ter frosting, showing that no hydrolytic activity had been instigated by the low temperature. There was, however, a considerable increase in glucoside content, as obtained by 4 hour autolysis. Thus the low temperature had caused the *production within the tissues of more cyanogenetic glucoside* than existed before the frosting.

Although furnishing but meager evidence, these two examples seem to show that the effects of anesthetics and of frost are very similar. The rupturing of cells by the frost is no doubt the primary cause for changed osmotic conditions and increased permeability, although, as in the case of other plants, lower temperatures may allow certain enzymes to gain the ascendency over those normally in control at other temperatures.

SUMMARY.

1. Sorghum leaves exposed to the vapors of chloroform, ether, and alcohol, yield more hydrocyanic acid, both glucosidic and non-glucosidic, than the normal leaves.

2. The anesthetics thus stimulate both the hydrolytic and the synthetic action of the glucoside enzymes.

3. This is submitted as a case of demonstrated enzyme synthesis *in vivo*.

4. Enzyme powder prepared from chloroformed leaves was about twenty-five times as active towards amygdalin as the powder from untreated leaves.

5. Frosting also causes an increased yield of both glucosidic and non-glucosidic hydrocyanic acid. This is partly due to a rupturing of the cells, and partly to disturbed enzyme equilibrium.

FACTORS INVOLVING THE ACCURACY OF CREATININE DETERMINATIONS IN HUMAN BLOOD.

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Considerable discussion has arisen in various laboratories, since the publication, by Gettler and Baker,[1] of the micro analysis of normal blood, as to what should be considered the true normal value for creatinine. Shall it be 0.1 to 0.5 mg. in 100 cc. of blood, as given by the above authors, or shall the values of Folin and Denis,[2] which are many times higher, be adhered to?

McCrudden and Sargent[3] have since published the statement: "In the light of these [McCrudden and Sargent's] experiments, it is clear that all that has been written hitherto concerning . . . . creatinine and creatine in the blood will have to be rejected altogether." They arrive at this conclusion as a result of an observation that a color similar to that obtained by creatinine on picric acid in the presence of sodium hydroxide is produced by the sodium hydroxide on picric acid alone. They furthermore give experimental proof that five-sixths of the total color produced in the method is due to the alkali, and only one-sixth to the creatinine.

Had these authors read our publication more closely, they probably could have used our creatinine figures to bear out part of their contention, and at the same time modify their statement with an exception. In making their sweeping statement, however, I must here point out that the creatinine values published by Gettler and Baker do not warrant their severe criticism. The sources of error which they mention, and many others which

[1] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.

[3] McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxvi, 527.

will be discussed in the experimental part, were recognized by us when we first started to use this method; they were quantitatively taken account of in all our work. That is why I now claim, after much experimental evidence in the laboratory, that the values of Gettler and Baker[1] are the correct ones, in preference to those of Folin and Denis.[2] It was Folin himself who called attention to the same effect of sodium hydroxide on picric acid. He even states that we can only then read our colorimeter with correct results, if the unknown color and the standard color are within one and a half times of each other.

In trying out this method previous to the determination of the creatinine values in normal blood,[1] we found at that time that Folin's suggestion was valid, but only for high values of creatinine (2 mg. in 100 cc. and over); when, however, it was a matter of estimating 0.1 to 0.5 mg. of creatinine in 100 cc. of blood, the above requirement was not sufficient. It was indeed necessary to have the standard and the unknown of the same depth of color, if we expected to get anywhere near the right value. To accomplish this in the simplest manner, we made up a series of standard creatinine solutions in picric acid, ranging from 0.1 to 2.1 mg. in 100 cc., as shown below. Each of these solutions we ran in the usual manner, adding oxalate and glucose in amounts as usually found in blood samples, and compared the colors in a Duboscq colorimeter with a M/72 dichromate solution. In this way we obtained a series of dichromate values, which were charted as follows:

Creatinine in 100 cc.	Placed at	M/72 bichromate reading.
mg.		
0.1	30	9
0.3	30	12
0.5	30	15
0.7	30	18
0.9	30	20
1.1	30	23
1.3	30	25
1.5	30	28
1.7	30	30
1.9	30	34
2.1	30	38

If now we compare an unknown (placed at 30) with this dichromate, and we obtain a reading of 15, the color of this reading is absolutely the same as given by 0.5 mg. of creatinine in 100 cc. It is evident that the unknown has thus been compared, indirectly, with a creatinine solution of the same strength.

The normal values of creatinine in blood are given as follows:

Folin and Denis[2]	1.1-1.4 mg. in 100 cc.
Myers and Fine[4]	1.0-2.0 " " 100 "
Gettler and Baker[1]	0.1-0.5 " " 100 "

It will be noticed that the values of Gettler and Baker are much lower than those of other authors; I attributed this difference at that time to our use of standards that were of the same depth as the unknown; but I made no quantitative investigation to prove the cause of this difference, and therefore laid no special stress upon it.

McCrudden and Sargent in their paper state that only one-sixth of the color, or $16\frac{2}{3}$ per cent, is due to creatinine, and five-sixths, or $83\frac{1}{3}$ per cent, to the alkali. They have determined this on a 0.5 mg. in 100 cc. creatinine solution. As far as they go, I have in the present investigation, been able to confirm their finding. But they have not investigated this point far enough. That alone would be no cause of error in the determination; for the comparison is made under similar conditions, with a standard creatinine solution in picric acid, and with exactly the same amount of alkali. Hence that same amount of additional color, due to the alkali, is obtained in the unknown as well as in the standard, and thus they cancel each other. The real cause for error McCrudden and Sargent do not call attention to. As will be shown in the experimental part, the lower the creatinine content the larger the percentage of color due to the alkali; on the other hand, the greater the concentration of creatinine the lower the percentage of color due to the alkali. Thus:

Concentration of creatinine, in 100 cc.	Color due to creatinine.	Color due to alkali
mg.	*per cent*	*per cent*
0.1	20	80
0.5	66	33
1.0	80	20
2.0	85	15

[4] Myers, V. C., and Fine, M. S., *Post-Graduate*, 1915, xxx, 39.

From this it is plain that if we compare a standard containing 1 mg. in 100 cc. with an unknown containing 0.1 mg., the 20 per cent of the color due to alkali will cancel 20 per cent of the 80 per cent color due to alkali in the unknown, and the difference, or 60 per cent of the color, still due to the alkali, will automatically add itself to the 20 per cent (real value) and give 80 per cent, a value four times too high. On the other hand, if we compare a standard and an unknown of the same color, say the 1 mg. value above, then the 20 per cent of alkali color in the standard will just cancel the 20 per cent of color due to alkali in the unknown, and the value of 80 per cent that is obtained is correct.

It is furthermore evident from the above table that the higher the creatinine value, the less does the percentage of color due to the alkali become, and hence the less the effect of this on the creatinine value obtained. Thus if we compare a 0.1 mg. solution with a 1.0 mg. standard the value obtained is three times (300 per cent) too high. While if we compare a 2.0 mg. solution with a 1.0 mg. standard the error is only about 5 per cent and this is not much more than the experimental error in reading the Duboscq colorimeter.

So, in general, for values above 2 mg. in 100 cc. that have been published by various other workers, the error is not sufficient to warrant their rejection, as McCrudden and Sargent suggest. For values less than 1 mg. in 100 cc. the error becomes appreciable, until at a 0.1 mg. concentration the values read are four to five times too high.

EXPERIMENTAL PART.

Since this article was sent for publication, Hunter[5] and also Folin[6] have shown that picric acid in solution on standing, exposed to light, deteriorates gradually, and there is then obtained a deep coloration on adding alkali, which pure picric acid does not give. Therefore, to assure those who are interested in creatinine determinations, I will state that in this work full account of any such deterioration has been taken. On treating 10 cc. of purest recrystallized saturated picric acid solution, used through-

[5] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 335.
[6] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

out this work, with 1 cc. of double normal NaOH, as in the method, and placing the same at 30 (Duboscq) the M/72 standard bichromate reads 7.5; using this same picric acid solution after standing in the laboratory from 3 to 4 months (not in direct sunlight) and treating in the same way, it read 8.0. I would therefore suggest that, as each stock solution of picric acid is made up, it be read as above; if the reading is between 7.5 and 8.0 it can be put in a brown glass bottle and used directly; if it reads more, purification must be resorted to.

Experiment I. Effect of Potassium Oxalate on Creatinine Determinations.

As the actual creatinine values were not desired here, but only to show the relative colors, a series of tubes containing increasing amounts of dichromate were set up, instead of using the Duboscq colorimeter. The first tube marked (0) was of the same color as picric acid alone. Each succeeding tube was made a little deeper in color by a common increment of 0.25 cc. of saturated dichromate solution. Thus the higher numbers represent deeper colors, the lower numbers represent less color.

With this series of colors, those obtained from varying amounts of creatinine, with varying amounts of oxalate were compared, with the results given in the following table.

5 cc. creatinine solution containing per 100 cc.	Saturated picric acid solution.	2 N NaOH per 10 cc.	Relative values found in presence of units of oxalate.				
			No oxalate.	Saturated oxalate.			
				0.1 cc.	0.5 cc.	1 cc.	2 cc.
mg.	*cc.*	*cc.*					
0.1	to 25	1	1	−1	0	0	0
0.25	" 25	1	3	2	0	0	0
0.5	" 25	1	7	5	0	0	0
1.0	" 25	1	19	11	6	5	4
2.0	" 25	1	30	18	11	9	10
3.0	" 25	1	+30	21	19	20	15
4.0	" 25	1	+30	27	24	25	21

Deduction.—Potassium oxalate has a strong bleaching effect on the picramic acid color, as seen in the tabulation. Passing from

Experiment IV—Concluded.

Creatinine per 100 cc. (Used 3 cc.)	Saturated picric acid solution containing 100 mg. of glucose in 100 cc.	Saturated potassium oxalate solution.	2 N NaOH per 10 cc.	Colorimetric readings after 10 min.	
				Standard M/24 bichromate.	Standard creatinine.*
mg.	*cc.*	*cc.*	*cc.*		
2.1	to 15	0.1	1	14	30
2.5	" 15	0.1	1	16	30
3.0	" 15	0.1	1	19	30
3.5	" 15	0.1	1	22	30
4.0	" 15	0.1	1	27	30
4.5	" 15	0.1	1	31	30
4.5	" 15	0.1	1	19	20
5.0	" 15	0.1	1	22	20
6.0	" 15	0.1	1	29	20
7.0	" 15	0.1	1	34	20
8.0	" 15	0.1	1	40	20
9.0	" 15	0.1	1	46	20
10.0	" 15	0.1	1	51	20

How to Use the Above Table.—Provided Folin's method is adhered to, as in the above, the unknown is placed at 30, and then the reading obtained on the colorimeter is translated, into mg. of *creatinine in 100 cc. of blood, directly by the figure in the first* column.

Experiment V. Comparing the Values Obtained by Folin's Standard with Those of Our Table.

Creatinine per 100 cc. (Used 3 cc.)	Saturated picric acid solution containing 100 mg. of glucose in 100 cc.	Saturated oxalate.	2 N NaOH per 10 cc.	Creatinine found by Folin's standard.	Creatinine by Gettler's table.
mg.	*cc.*	*cc.*	*cc.*		
0.1	to 15	0.1	1	0.6	0.1
0.3	" 15	0.1	1	0.6	0.35
0.5	" 15	0.1	1	0.7	0.5
0.7	" 15	0.1	1	0.9	0.7
0.9	" 15	0.1	1	1.0	0.95
1.1	" 15	0.1	1	1.0	1.1
1.3	" 15	0.1	1	1.1	1.3
1.5	" 15	0.1	1	1.2	1.4
1.7	" 15	0.1	1	1.3	1.6
1.9	" 15	0.1	1	1.5	1.8
2.1	" 15	0.1	1	1.6	2.1

The above table shows plainly that for low values, as found in normal blood, the readings by the Folin standard are much too high. When the values reach 1 mg., as in the standard,[7] then only does the Folin standard give the correct value. Finally, if the unknown has a higher content than the standard, the values always run low. By means of the standardized table, however, the values obtained agree very closely with the amounts actually present.

Experiment VI. Some Additional Normal Creatinine Values.

No.	Name.	Reading of colorimeter.	Creatinine per 100 cc.
			mg.
1	Mc	10	0.15
2	H	9	0.1
3	D	8	0:1
4	Sh	8	0.1
5	K	9	0.1
6	G	13	0.4
7	T	12	0.3
8	J	9	0.1
9	R	10	0.15
10	W	11	0.2
11	E	8	0.1

Deduction.—The normal value for creatinine in blood is 0.1 to 0.5 mg. in 100 cc. This confirms the values of Gettler and Baker. The values of Folin and Denis[2] and of Myers and Fine[4] are much too high.

SUMMARY.

I. In the light of the experimental evidence just presented, I cannot subscribe to McCrudden and Sargent's view, that all creatinine blood values heretofore reported are useless, because of error in method. The values, in normal blood, of Gettler and Baker[1] are correct and are here again confirmed. Furthermore,

[7] Folin's standard is 0.2 mg. of creatinine in 100 cc. Of this 10 cc. are used and colorized without diluting. It must be remembered, however, that the blood sample is diluted fivefold, so that the standard, calculating on a similar dilution, represents 1 mg. in 100 cc.

in all other reported values, which lie at 2 mg. in 100 cc. and above the error is not enough to warrant rejection.

II. The method for estimating creatinine in blood has been critically studied as to the following: (*a*) Oxalate has a strong bleaching action on the picramic acid color, so that care must be taken not to add too much. (*b*) Standing longer than 10 minutes brings in a large error, because color changes rapidly thereafter. (*c*) The total color that is read in the colorimeter is not all due to creatinine. At 0.1 mg. concentration it is only about 23 per cent of the total. As the creatinine content increases the percentage of color due to it increases, so that at a 2.0 mg. concentration it is 83 per cent and beyond that it more and more approaches 100 per cent.

III. A table is given for reading mg. of creatinine in 100 cc. of blood directly, in which all the above sources of error have been accounted for.

IV. Known solutions of creatinine have been determined with the Folin standard and also with this table, conclusively proving that for values of 0.1 to 0.5 mg. of creatinine the Folin standard gives values much too high.

V. Eleven additional normals have been determined for their creatinine value. These range between 0.1 and 0.4 mg. in 100 cc., confirming Gettler and Baker's previous report.

The values of 1.0 to 2.0 mg. in 100 cc. as reported by Folin and Denis, and by Myers and Fine. are much too high.

THE BEHAVIOR OF CHICKENS FED RATIONS RESTRICTED TO THE CEREAL GRAINS.*

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PLATE 1.

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With the prevailing interest in problems of animal nutrition some confusion will probably arise if investigators translate without reserve and without definite knowledge the results secured with laboratory animals to those of economic value or to man himself. In investigations carried on from this laboratory, involving rats, swine, and cattle, there has been constant agreement among these three species in the effects produced by the several rations studied.

There may have been some slight differences between the resistances of swine and rats to certain rations involved in our extended inquiries, but on the whole substantial agreement has resulted. It does not follow, however, that this would be true with every ration that may arise for investigation, nor should we expect the requirements for the normal factors of nutrition to be exactly identical among all species of mammals. For purposes of a definite record of the behavior of different species we desire to record here the fact that chickens behave entirely differently from either rats or swine on rations limited to the corn (maize) or wheat grains and their products.

With swine or rats[1] there is practically no growth when the ration is restricted to either a corn meal, gluten feed mixture—or a wheat meal, wheat gluten ration. On the corn meal, gluten feed ration swine will maintain themselves for a long time—at

* Published with the permission of the Director of the Agricultural Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.

least several months—without evidences of serious malnutrition; ultimately, however, their coats will show some roughness, the appetite will wane, and slight stiffness may arise, but no serious pathological symptoms develop, at least in the periods under our observation, 6 months. However, there will be little if any growth and no reproduction. When a suitable salt mixture is added to the corn grain ration, growth at a fair rate and continued well-being result. Reproduction likewise occurs, although it will not be normal.

Where wheat, wheat gluten forms the ration pathological conditions will manifest themselves with swine or rats in 4 to 5 months. Maintenance fails and marked symptoms of nutritional derangement develop. In the presence of a salt mixture there may be some early growth, but after a few months' restriction to this ration loss in weight sets in accompanied by important histological changes in the spinal cord.[2]

With chickens, starting at 3 to 4 pounds' weight, no such results as described above for mammals were obtained, and our observations extended over periods of 7 months' duration and in some cases for 12 months. On either the corn, gluten feed, or wheat, wheat gluten rations, these animals will make slow growth, but what is more important, they will reach maturity, maintain themselves without pathological symptoms developing, and produce fertile eggs. With swine or rats there is complete cessation of the œstrum on rations limited to either of these two grains, fortified only with the protein concentrate from the same grain.

With the chickens whose records are here given there was in many cases a fall in weight at the time of the laying period, and not enough time elapsed between the termination of the laying period and the termination of the experimental observations to regain the maximum weight. There was, however, variation among the individuals in this respect and a considerable number showed no such fall in weight during the laying period. It is to be expected that under unfavorable environmental conditions, such as strict housing and limited quarters, variations such as these would occur. The same results, however, may be obtained

[2] Hart, E. B., Miller, W. S., and McCollum, E. V., *J. Biol. Chem.*, 1916, xxv, 239.

with chickens kept under normal conditions and fed standard rations.

To all the rations used in these studies there was an addition of calcium carbonate to provide for egg shell production. This was a modification in the ash mixture of the ration and might be considered a crucial factor in the slow growth, maintenance, and reproduction secured with this species; nevertheless we have added to a wheat, wheat gluten ration calcium lactate, which should have modified the ration in the same way as here modified, but with absolute failure where swine were involved in the inquiry.

We have numerous records of failure with mammals where wheat served as a large proportion of the diet and have attributed such failure to the inherent toxicity in the grain.[3] Such disaster will result even where the animals (swine) are placed on the ration after reaching a weight of 75 to 100 pounds. This fact should eliminate any criticism that the success with chickens—limited to a wheat grain, wheat gluten, calcium carbonate ration—was made possible by the choice of birds already somewhat passed the half grown mark. Such toxicity as occurs in the wheat grain had no apparent detrimental effect on chickens.

We know, of course, that it is impossible to place baby chicks upon such rations as here used and have a successful rearing. It is possible, however, to take this species at the half grown period and obtain slow growth and fertile egg production. At what age or weight they can be started on such rations and secure growth and egg production is yet to be determined.

Perhaps we should not wonder at such results. It is apparent from paleontological records that this species (chickens) occupies a lower position in the scale of animal evolution than mammals, and the quantitative requirements of the normal factors for nutrition, established for a mammal, may not be exactly like those required by a fowl. It is certain from the data here presented that its mineral requirements for slow growth or maintenance are not like those of either the rat or pig, especially under conditions of a poor adjustment in the ration of the other factors for normal

[3] Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105. Hart, Miller, and McCollum, *ibid.*, 1916, xxv, 239.

nutrition. We have, however, seen pigs[2] grow well on a grain diet, fortified with a protein concentrate like oil meal *and an addition of but 1 per cent of the ration as meat scraps.* The latter, in the proportion used, could not possibly have modified the mineral content of the ration in any important degree, but without its presence failure resulted. By what action such a small percentage of meat could turn a ration from a failure into a success for growth in mammals needs specific investigation. The extreme exponents of the vitamine theory would find a ready explanation in the cry of "deficiency," but that is not the explanation. Substituting butter fat for the meat does not make the ration a success.

What the differences are between species in respect to the requirements of the unidentified dietary factors A and B cannot be definitely decided from these data. But it is true that the two grains, corn or wheat, provide chickens with enough of these substances at the half grown period for continued slow growth, maintenance, and production of fertile and hatchable eggs, if we take the records of maximum performance; but future work must determine what are the requirements in respect to these factors by baby chicks making for normal growth and reproduction. We are assuming that this species cannot synthesize these unidentified dietary factors, an assumption which is undoubtedly correct, especially in reference to the unidentified factor B.

Osborne and Mendel[4] have recently pointed out that the same relation holds for chickens as for rats in respect to their dependence for normal growth on the qualitative make-up of the proteins. The chicken's tolerance for wheat, without an adjustment of the nutritive factors of this grain, as is necessary with swine or rats, is evidence of a difference in its metabolism as compared with that of the mammal—a fact also supported by the well known differences between their purine metabolism.

EXPERIMENTAL.

For both seasons of experimental work vigorous pullets (Rhode Island Reds) were selected. There were three in each lot, confined to wire cages with shavings as litter and scratch. They

[4] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1916, xxvi, 293.

were placed in the cages November 1 and the experimental observations terminated June 1, a period of 7 months' observation. New birds were selected for the 2nd year; distilled water was used for all lots with the exception of one of the controls where well water was used. Quartz grits were used in the hoppers. The salts were mixed intimately with the feeds whether fed as dry or wet mash. Calcium carbonate was used as the precipitated carbonate in the proportion of 3 pounds to 100 pounds of air-dried feed. The protein concentrate was used either as dry or wet mash, but mixed with some of the cereal grain. In addition, some of the whole grain was cracked and used as a scratch feed. The proportion of grain to concentrate as indicated in the table was, however, always kept constant over any given number of days of consumption. This was accomplished by weighing out the proportion given in the table and then seeing to it that each proportion was fed and consumed before a new batch of material was started.

The feeds used in the check lots consisted of three parts of corn, two parts of wheat, one part of oats as scratch, and one part each of bran, middlings, and corn as mash. These were supplied at the rate of two parts of scratch feed to one of mash. The check ration is by no means a very successful one for rapid growth and large production with chickens, but what it accomplished as contrasted with its effects on swine or rats is especially what we want to emphasize. It would lead to absolute disaster with swine or rats, but with chickens it gave moderate growth and reproduction. It should be kept in mind that this ration was of strictly vegetable origin derived wholly from cereal grains and that it maintained this species with production of fertile eggs. In fact during the short laying period of 2 months exhibited by these birds (March 1 to May 1) there was averaged an egg per fowl, every 3rd day.

In addition to our studies of the effects of restricted corn and wheat rations on the growth and egg-laying production of chickens we have extensive records where the rations were restricted to oat grain or to barley grain and their products. No detailed discussion of these records will be given at this time, but the statement can be made that this species could maintain itself and pro-

duce fertile eggs on rations limited to these two sources as well as on corn or wheat grain and their products.

During the 2nd year of observation the records of egg production represent those collected and not those produced. Most of the eggs were broken and in some cases eaten. These differences between the two records in the 2 years were largely due to the fact that a new attendant, unaccustomed to the work, was in charge the 2nd year. The size of the eggs produced in either year was normal and there was no appreciable variation among lots in their fertility. The hatching power—the percentage of live chickens produced from a given number of eggs—was a normal figure.

Addition to either a corn grain, corn gluten or wheat grain wheat gluten ration of a salt mixture, which at least with corn makes possible the growth of swine, here made little if any improvement in the ration.

The addition of butter fat and salts or of butter fat, salts, and casein likewise gave little if any improvement over rations not carrying these additions. It might be contended that these were practically maintenance experiments, but a study of the data will show that most of the birds made considerable gains in weight—15 to 25 per cent of their initial weight and some even more than this. Birds 179 and 529—wheat, wheat gluten, calcium carbonate ration—made increases of 30 and 40 per cent respectively over their initial weight. Approximately similar increases were made on the corn, gluten feed, calcium carbonate ration.

All these rations gave low egg production; we are not contending that they cannot be improved, because as a matter of fact they actually are improved in practice by the addition of meat scraps, granulated milk, free range, etc., but that they accomplished anything as contrasted with their effect upon a mammal is the point we wish to emphasize.

Some of the birds pulled their feathers, but this occurred more or less in all lots. Figs. 1 to 7 show the condition of some of these animals at the termination of the experiment. There was absolutely no indication of polyneuritis among the birds on any of these rations. For comparison, see an earlier illustration of a pig on a wheat, wheat gluten, salt mixture ration.[5] This species

[5] Hart and McCollum, *J. Biol. Chem.*, 1914, xix, 373, Fig. 1.

TABLE 1.

Record of Results, 1914–15. Duration, November 1 to June 1. Laying Period March 1 to May 1.

Ration.	Bird No.	Weight.			Egg production.	Fertility.	Hatching power.
		Initial.	Maximum.	Final.			
		lbs.	*lbs.*	*lbs.*		*per cent*	*per cent*
Corn meal 70 lbs. Gluten feed 30 lbs. $CaCO_3$ 3 lbs. Quartz.	171 172 173	3.9 3.6 3.9	4.7 4.6 4.75	3.8 3.7 4.15	27	100	50
Corn meal 70 lbs. Gluten feed 30 lbs. $CaCO_3$ 3 lbs. K_2HPO_4 323 gm. Ca lactate 513 gm. Quartz.	174 175 176	3.9 3.2 4.0	5.25 3.9 4.8	5.25 2.35 3.45	36	90	60
Corn 95.5 lbs. Casein 2.5 lbs. K_2HPO_4 323 gm. Ca lactate 513 gm. $CaCO_3$ 3 lbs. Quartz.	189 190 191	4.2 3.1 3.5	4.5 3.8	4.0 2.15 3.35	34	100	60
Wheat meal 95.5 lbs. Wheat gluten 2.5 lbs. $CaCO_3$ 3 lbs. Quartz.	177 178 179	3.8 3.3 3.6	4.3 3.5 4.6	4.0 3.15 4.15	27	100	80
Wheat 95.5 lbs. Wheat gluten 2.5 lbs. K_2HPO_4 323 gm. Ca lactate 513 gm. $CaCO_3$ 3 lbs. Quartz.	180 181 182	2.9 3.4 3.7	 4.5	1.3 1.85 4.35	33	90	90
Wheat 95.5 lbs. Casein 2.5 lbs. K_2HPO_4 323 gm. Ca lactate 513 gm. $CaCO_3$ 3 lbs. Quartz.	183 184 185	3.7 3.1 3.7	4.3 3.6 4.85	2.55 2.8 4.35	35	100	80

TABLE I—*Concluded.*

Ration.	Bird No.	Weight. Initial.	Weight. Maximum.	Weight. Final.	Egg production.	Fertility.	Hatching power.
		lbs.	*lbs.*	*lbs.*		*per cent*	*per cent*
Wheat 95.5 lbs.	186	3.6	4.65	4.35	32	80	80
Casein 2.5 lbs.	187	3.3	5.25	4.85			
Butter fat 2 lbs.	188	3.9	5.35	3.75			
K_2HPO_4 323 gm.							
Ca lactate 513 gm.							
$CaCO_3$ 3 lbs.							
Quartz.							
Check, variety grain ration.	192	3.2	4.6	4.05	69	100	90
Distilled water.	193	3.5	4.5	4.0			
$CaCO_3$ 3 lbs.	194	3.8	4.75	3.9			
Quartz.							
Check, variety grain ration.	195	3.5	4.25	3.7	61	80	60
Well water.	196	3.2	4.3	3.7			
$CaCO_3$ 3 lbs.	197	4.4	5.85	4.25			
Quartz.							

TABLE II.

Record of Results, 1915–16. Duration, November 1 to June 1. Laying Period, March 1 to May 1.

Ration.	Bird No.	Weight. Initial.	Weight. Maximum.	Weight. Final.	Eggs collected.*
		lbs.	*lbs.*	*lbs.*	
Corn meal 70 lbs.	521	5.4	6.8	4.6	4
Gluten feed 30 lbs.	522	3.3	4.8	3.6	
$CaCO_3$ 3 lbs	523	4.5	5.1	3.4	
Quartz.					
Corn meal 70 lbs.	524	3.7	5.7	4.5	5
Gluten feed 30 lbs.	525	4.7	5.8	4.0	
K_2HPO_4 323 gm.	526	3.3	4.9	2.7	
$CaCO_3$ 3 lbs.					
Ca lactate 513 gm.					
Quartz.					

* Eggs collected. Most of them were broken in the nest or eaten.

TABLE II—*Concluded.*

Ration.	Bird No.	Weight. Initial.	Weight. Maximum	Weight. Final.	Eggs collected.*
		lbs.	*lbs.*	*lbs.*	
Corn 95.5 lbs.	539	4.0	5.6	4.0	1
Casein 2.5 lbs.	540	3.3	4 8	3.6	
$CaCO_3$ 3 lbs.	541	3.8	4.8	4.3	
K_2HPO_4 323 gm.					
Ca lactate 513 gm.					
Quartz.					
Wheat meal 95.5 lbs.	527	4 8	5 9	3.2	0
Wheat gluten 2.5 lbs.	528	3 7	4 5	3 4	
$CaCO_3$ 3 lbs.	529	3 4	4.8	4 0	
Quartz.					
Wheat meal 95.5 lbs.	530	4 5	5 6	4 3	1
Wheat gluten 2.5 lbs.	531	4.7	4 8	4 0	
$CaCO_3$ 3 lbs.	532	3.6	4.8	3.1	
K_2HPO_4 323 gm.					
Ca lactate 513 gm.					
Quartz.					
Wheat 95.5 lbs.	533	4 9	6 4	4 7	9
Casein 2.5 lbs.	534	3.5	4 6	2.6	
$CaCO_3$ 3 lbs.	535	3.9	5.6	4 3	
K_2HPO_4 323 gm.					
Ca lactate 513 gm.					
Quartz.					
Wheat 95.5 lbs.	536	5 1	7.0	4.0	5
Casein 2.5 lbs.	537	3 4	5 3	4 0	
Butter fat 2 lbs.	538	3 1	4 2	3 0	
$CaCO_3$ 3 lbs.					
K_2HPO_4 323 gm.					
Ca lactate 513 gm.					
Quartz.					
Check, variety grain ration.	542	4 3	5 7	4 8	8
Distilled water.	543	3 6	5 8	4.4	
$CaCO_3$ 3 lbs.	544	3 3	5 0	3.7	
Quartz.					
Check, variety grain ration.	545	3 5	5 0	4 7	0
Well water.	546	3 9	6 2	4 9	
$CaCO_3$ 3 lbs.	547	3 6	5.3	4.2	
Quartz.					

* Eggs collected. Most of them were broken in the nest or eaten.

tolerates this ration for a few months, but ultimately will begin to lose weight and pass into a miserable condition.

SUMMARY.

1. Chickens started at half the normal weight can make slow growth, maintain themselves, and produce fertile eggs on rations limited to corn meal, gluten feed, and calcium carbonate, or wheat meal, wheat gluten, and calcium carbonate.

2. These results are in marked contrast to our records with swine or rats where these rations lead to loss of weight, cessation of œstrum, and with wheat to a condition resembling polyneuritis.

3. It is apparent that the mineral requirements at least, and possibly the requirements for the other normal nutritive factors are not the same for chickens as they are for mammals. Further. the chicken's ability to tolerate, without disaster and withou modification of the ration, the toxic material of wheat speaks fo a metabolism distinct from that of swine or rats.

4. Where half grown chickens were used there was no importan improvement in the rates of growth or egg-laying capacity by supplementing the grains with either salts, casein, or butter fat, or a combination of the three, as contrasted with the results secured with the grain, grain protein concentrate, $CaCO_3$ ration. The protein level in all cases was approximately 12 per cent. On all of these rations the number of eggs produced, although fertile, was limited. This fact, at variance with the best results of practice where animal protein concentrates have proved of great value as supplements to cereal grains for a large egg production, would suggest that either the higher plane—20 to 25 per cent—of protein intake of superior quality secured in practice by the use of the animal protein concentrates (meat scraps, milk, etc.) explains these differences, or else the latter contribute certain factors necessary for large egg production which the cereal grain concentrates or casein fail to carry. The matter needs further investigation.

EXPLANATION OF PLATE 1.

FIG. 1. Condition of a fowl at the end of 7 months on a ration of 95.5 pounds wheat meal, 2.5 pounds wheat gluten, 3 pounds $CaCO_3$, quartz, and distilled water. This animal was in as good condition as were fowls receiving the wheat meal plus casein, butter fat, and a salt-mixture (see Fig. 3). Contrast this figure with the condition of a mammal receiving a similar ration.[5] This ration is fatal to a mammal.

FIG. 2. After 7 months on a ration of 95.5 pounds wheat meal, 2.5 pounds casein, 3 pounds $CaCO_3$, 323 gm. K_2HPO_4, 513 gm. calcium lactate, quartz, and distilled water. The animal was in splendid condition.

FIG. 3. After 7 months on a ration of 95.5 pounds wheat meal, 2.5 pounds casein, 2 pounds butter fat, 3 pounds $CaCO_3$, 323 gm. K_2HPO_4, 513 gm. calcium lactate, quartz, and distilled water. Apparently normal condition, although some of the feathers had been pulled. This fowl was in no better condition than those receiving the wheat ration, but without casein, butter fat, or the salt mixture (see Fig. 1).

FIG. 4. Photographed at the end of 7 months on a ration derived wholly from seeds; scratch, 40 pounds corn, 40 pounds wheat, 20 pounds oats; mash, 33 pounds bran, 33 pounds middlings, 33 pounds corn meal, distilled water, and quartz. Fed two parts of scratch to one of mash. A normal fowl in every respect. $CaCO_3$ was not present in this ration. Such a ration would terminate growth in a mammal (pig or rat) and induce loss in weight and serious pathological changes.

FIG. 5. After 7 months on a ration of 70 pounds corn meal, 30 pounds gluten feed, 3 pounds $CaCO_3$, distilled water, and quartz. From all appearances this was a normal fowl.

FIG. 6. On the following ration for 7 months: 70 pounds corn meal, 30 pounds gluten feed, 3 pounds $CaCO_3$, 323 gm. K_2HPO_4, 513 gm. calcium lactate, distilled water, and quartz. Appeared in better condition than the individual shown in Figs. 5 or 7. These differences are really due to feather picking exhibited more by some individuals than by others.

FIG. 7. Photographed after 7 months on a ration of 95.5 pounds corn meal, 2.5 pounds casein, 3 pounds $CaCO_3$, 323 gm. K_2HPO_4, 513 gm. lactate, distilled water, and quartz. In good physical condition, but had pulled feathers.

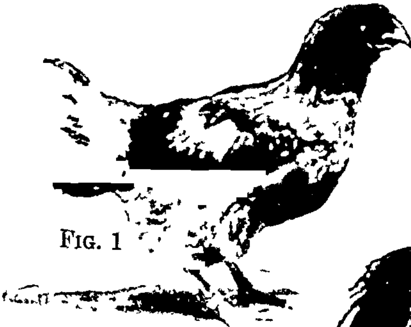

FIG. 1

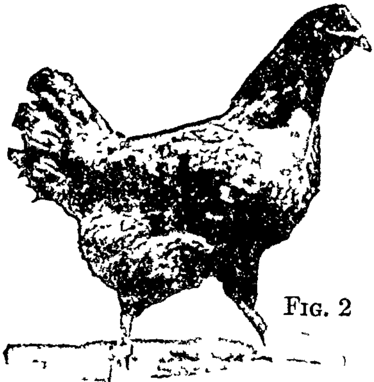

FIG. 2

FIG. 3

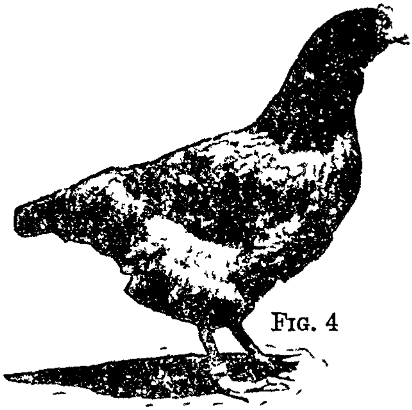

FIG. 4

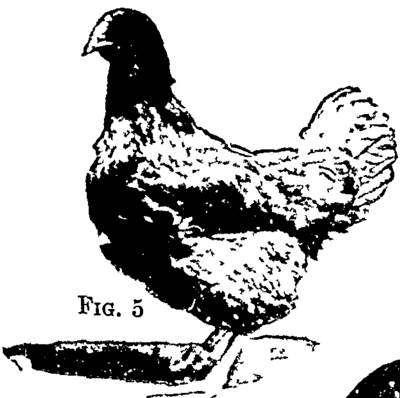

FIG. 5

FIG. 6

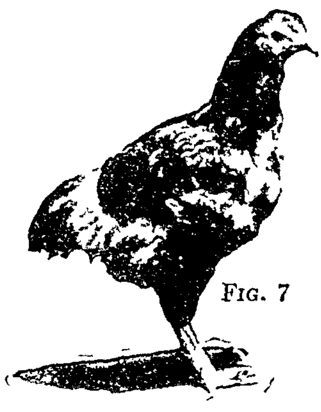

FIG. 7

THE RELATIVE VALUE OF CERTAIN PROTEINS AND PROTEIN CONCENTRATES AS SUPPLEMENTS TO CORN GLUTEN.*

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It has become more and more evident for some time that the question of the protein requirement, particularly in the case of farm animals, is in need of critical review if not of thorough revision. Practical experience has long since demonstrated that certain of the vegetable proteins, such as those of the maize kernel, fail to promote growth effectively unless they are supplemented by added nutrients. The deficiencies may be of more than one sort; but among them the inadequate or qualitatively inappropriate character of the protein is a clearly demonstrated feature. The evidence which has lately been offered for the dietary need of suitable amino-acids that cannot be synthesized by the animal organism has directed emphasis to the real significance, from the standpoint of the protein requirement, of supplementing one food by another containing a different variety of proteins.[1] It has become clearer that such inadequate but perhaps cheap proteins can be supplemented advantageously by one which supplies the needed factors, *i.e.*, amino-acids. Thus in attempting to improve a diet of which corn or corn meal forms the chief constituent our experiments have indicated that better results would be obtained if the added protein concentrate furnished

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Mendel, L. B., Harvey Society Lectures for 1915–16; also *J. Am. Med. Assn.*, 1915, lxiv, 1539.

protein rich in tryptophane and lysine.[2] *Hitherto animal fodders have been valued largely on the basis of the amount* of protein which they contain, little attention being paid to the qualitative character of the protein. The principle that the quality of the protein should be considered in order to make sure that the ration is not deficient in any of the essential amino-acids is beginning to find expression in the most recent books on the feeding of farm animals,[3] but suitable directions for its application in practice remain to be formulated. Inasmuch as differences exist in the economy of correcting different inadequate protein foods with appropriate supplementary proteins, one problem involves the discovery of suitable readily available mixtures which shall not have any serious relative shortage of any essential amino-acid group.

Indications of the possibilities in this field of investigation have already been furnished by our earlier experiments with growing rats.[4] Thus experiments with additions of other proteins to corn gluten, containing a mixture of corn proteins with which only very slow growth is possible in rats even when all other (non-protein) essential dietary components are supplied, have shown results in accord with what one might expect from the presumable amino-acid make-up of the proteins added. Lactalbumin, rich in both tryptophane and lysine, appears to be a most efficient adjuvant; casein or edestin must be added in far larger proportion to accomplish results approaching those of the lactalbumin. In experiments to determine the relative values for milk production of different supplements to corn, Hart and Humphrey[5] found

[2] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1914, xvii, 325; 1914, xviii, 1; 1916, xxv, 1; 1916, xxvi, 293. See also Hogan, A. G., *ibid.*, 1916, xxvii, 193. The initial failure of Hogan to cause any improvement in the growth of rats by additions of lysine and tryptophane to his basal corn ration was presumably due to some deficiency in the diet other than the protein factor, making it impossible for the beneficial effect of the amino-acids to manifest itself. The effect of any specific addition to a ration can only be determined when all the other conditions are known to be satisfactory.

[3] Cf. Bull, S., The Principles of Feeding Farm Animals, New York, 1916, pp. 82, 131.

[4] Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 1.

[5] Hart, E. B., and Humphrey, G. C., *J. Biol. Chem.*, 1915, xxi, 239; 1916, xxvi, 457.

that the milk proteins had a percentage efficiency of about 60, oil meal 61, distillers' grains 60, gluten feed 45, corn 40, and wheat 36.

To obtain a further survey of the possibilities of measuring the comparative nutritive potencies of different proteins or protein concentrates by the methods of feeding which we have pursued successfully for some years with rats and more recently with chickens,[6] we have undertaken a large number of additional experiments. Most of them involve the question of growth on diets made adequate in all details except the protein used. In this respect they differ from most earlier feeding trials with farm animals. In one series with a liberal supply of inadequate corn gluten as the basis of the nitrogenous part of the ration, diets were made with combinations containing varying proportions of different proteins, either isolated or in the form of protein fodder "concentrates," but in which the total amount of protein was substantially the same. These experiments soon showed that the increments of body weight may vary very widely with the *proportion* of any particular protein product added; or they may show variations related to the *same proportion of different proteins*, in an otherwise unchanged food mixture. For example, if rations containing a fixed proportion of total protein—about 16 per cent in the case of our standard foods—are prepared with increasing proportions of a protein like lactalbumin or casein and correspondingly decreasing amounts of the basal corn gluten, the effect is clearly reflected in the character of the growth which they afford. Occasionally, however, an animal will eat so much more of a food relatively inferior, from a chemical standpoint, that the *absolute* intake of the preferable protein will be comparatively large despite its relative paucity, *i.e.*, low percentage content, in the food mixture.

It was pointed out in an earlier paper[7] that no strict comparison can be made between the different proteins unless the total calorie intake, the absolute amount of protein eaten, the quantities of inorganic salts, and amount of "food accessories" are uniform. Strict comparisons of the nutritive value of proteins in

[6] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 293.
[7] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 1.

growth will therefore only be trustworthy when the experiments are comparable with respect to the total amount of such a food eaten during a definite period of time.

Experiments with carefully *limited* daily feeding in the manner described in the earlier paper are exceedingly laborious. We have therefore conducted many further trials in which the food mixtures containing unlike combinations of different proteins were offered *ad libitum* to rats. The proteins and protein concentrates used in these experiments are as follows:

1. *Casein.*—Prepared in this laboratory by precipitation from separator milk with hydrochloric acid, and subsequently purified by several reprecipitations and extractions with alcohol.

2. *Lactalbumin.*–Prepared in this laboratory by heat coagulation of the filtrate from the casein precipitate.

3. *Edestin.*—Prepared in this laboratory by extraction of ground hemp seed with sodium chloride and repeated solution and precipitation by dilution and cooling.

4. *Cottonseed Protein.*—Prepared in this laboratory by extraction of cottonseed flour with sodium hydroxide and precipitation by neutralization.

5. *Corn Gluten.*—Prepared for us by the Corn Products Refining Co.[4] Nitrogen content = 6.4 per cent ($N \times 6.25 = 40.0$ per cent protein).

6. *"Milkalbumin."*—A commercial product containing 8.5 per cent of nitrogen ($N \times 6.38 = 54.2$ per cent of protein) and 23.8 per cent of ash.

7. *Cottonseed Flour.*—From the Schulenburg Oil Mills, Schulenburg, Texas. Nitrogen content = 8.1 per cent ($N \times 5.4 = 43.7$ per cent protein).

8. *Soy Bean Flour.*—From the Cereo Co., Tappan, N. Y. Nitrogen content = 8.05 per cent ($N \times 5.88 = 47.3$ per cent protein).

9. *Beef Tissue.*—Prepared in this laboratory by drying the residue remaining after thoroughly extracting fresh chopped beef with water. Nitrogen content = 14.3 per cent ($N \times 6.25 = 89.4$ per cent protein).

10. *Fish Meat Meal.*—A commercial product made from herring and obtained from the United States Department of Agriculture. Nitrogen content = 9.68 per cent ($N \times 6.25 = 60.5$ per cent protein).

11. Corn Oil Cake.—A preparation of corn germs obtained from a distillery in Peoria, Ill. Nitrogen content = 2.8 per cent (N $\times$ 6.25 = 17.5 per cent protein).

12. "Vegetable Albumin Flour."—A product rich in gliadin, obtained from Arthur S. Hoyt Co., New York, and presumably consisting of the residues from the manufacture of wheat starch. Nitrogen content = 13.1 per cent (N $\times$ 5.7 = 74.7 per cent protein).

13. Brewers' Grains.—From a brewery in Milwaukee. Nitrogen content = 7.2 per cent (N $\times$ 6.25 = 45.0 per cent protein).

14. Pea Meal.—Prepared in this laboratory by grinding dry peas to a fine powder. Nitrogen content = 4.5 per cent (N $\times$ 5.88 = 26.5 per cent protein).

15. Peanut Meal.—A preparation of ground peanuts from which the oil had been expressed, obtained from the United States Department of Agriculture. Nitrogen content = 6.6 per cent (N $\times$ 5.88 = 38.8 per cent protein).

16. Distillers' Grains.—From a distillery in Peoria, Ill. Nitrogen content = 4.7 per cent (N $\times$ 6.25 = 29.4 per cent protein).

The gains in weight, which we have used as the most suitable index of growth, show wide variations for the different protein mixtures. As an illustration we may cite the following data, from substantially all of our numerous protocols, to indicate the growth of male albino rats in 42 days from an initial body weight of 60 gm. (corresponding to about 44 days of age). The food mixtures in all of the trials reported in this paper had approximately the following composition, unless otherwise indicated:

	per cent
Protein	16
"Protein-free milk"	28
Starch	28
Butter fat	18
Lard	10

Although the general trend of these results points to marked differences in the capacity of different proteins to supplement this preparation of corn gluten for the uses of growth, the figures are too variable and ill defined to permit tenable generalizations unless the conclusions are compiled from large statistical material. The wide variations with the same rations are usually due, as could easily be demonstrated from our records of the food intake, to

TABLE I.

Showing Gains of Body Weight in 42 Days From an Initial Body Weight of 60 Gm. on Otherwise Comparable Corn Gluten Food Mixtures Containing Various Protein Supplements.

Concentration of the proteins in the food.		Rat.	Gained in 42 days.
per cent	*per cent*		*gm.*
Corn gluten 15.3		1350♂	24
" "		1347♂	22
Corn gluten 11.4 + lactalbumin	3.7	1445♂	106
" " "		1424♂	98
" " "		1432♂	92
" " 12.7 "	2.5	3280♂	66
" " 12.8 "	2.1	3276♂	48
" " 13.0 "	1.9	3330♂	49
" " "		3428♂	29
" " "		3425♂	29
" " "		3340♂	26
Corn gluten 10.2 + "milkalbumin"	5.8	2111♂	102
" " "		2109♂	89
" " 11.4 "	4.4	2045♂	74
" " "		2032♂	60
Corn gluten 11.7 + casein	4.1	3423♂	65
" " "		3433♂	62
" " "		3285♂	55
" " "		3292♂	22
" " 13.0 "	2.7	3527♂	51
" " "		3575♂	36
" " "		3517♂	35
Corn gluten 7.8 + edestin	8.4	3286♂	48
" " "		3438♂	29
" " "		3370♂	28
" " "		3424♂	18
" " 11.7 "	4.2	3296♂	41
" " "		3289♂	28

TABLE I—*Continued.*

Concentration of the proteins in the food.		Rat.	Gained in 42 days.
per cent	*per cent*		*gm.*
Corn gluten 7.8 + cottonseed protein	7.2	3576♂	54
" " " "		3518♂	36
" " 10.4 " "	4.8	3394♂	44
" " " "		3393♂	39
" " 11.7 " "	3.6	3390♂	43
" " " "		3396♂	43
Corn gluten 7.8 + cottonseed flour	9.0	3268♂	103
" " " "		3267♂	74
" " 10.1 " "	6.0	3290♂	46
" " " "		3314♂	46
" " 11.4 " "	4.4	3367♂	58
" " " "		3369♂	51
" " " "		3429♂	44
" " " "		3435♂	24
" " 12.5 " "	3.6	3513♂	47
" " " "		3522♂	36
Corn gluten 7.6 + soy bean flour	9.0	3395♂	112
" " " " "		3403♂	85
" " 10.4 " " "	6.0	3455♂	111
" " " " "		3457♂	94
" " 11.4 " " "	4.5	3443♂	38
" " " " "		3458♂	36
Corn gluten 10.4 + beef tissue	5.4	3391♂	59
" " " "		3399♂	52
" " 11.7 " "	4.0	3325♂	44
" " " "		3405♂	44
" " " "		3323♂	36
" " 12.2 " "	3.2	3309♂	66
" " " "		3324♂	37

TABLE I—*Concluded.*

Concentration of the proteins in the food.		Rat.	Gained in 42 days.
per cent	*per cent*		*gm.*
Corn gluten 10.4 + fish meat meal	6.1	3326♂	54
" " " " "		3310♂	42
Corn gluten 11.2 + corn oil cake	4.6	3331♂	49
" " " " "		3339♂	41
Corn gluten 5.4 + "vegetable albumin flour"	10.5	3417♂	23
" " " " "		3422♂	22
" " 7.6 " " "	7.5	3263♀	26
" " " " "		3264♀	16
Corn gluten 7.6 + brewers' grains	9.1	3332♂	36
" " " "		3311♂	31
" " 10.4 " "	5.9	2815♀	31
" " " "		2821♂	30
" " " "		2809♂	25
Corn gluten 7.8 + pea meal	7.9	3553♂	79
" " " "		3486♂	53
Corn gluten 7.8 + peanut meal	8.2	3540♂	98
" " " "		3491♂	53

the unlike food consumption of individual animals. In a general way it is nevertheless apparent that among the proteins studied small addenda of lactalbumin far surpass in their supplementing efficiency decidedly larger proportions of other proteins or high protein feeds tested. Characteristic examples of supplementary inadequacy, on the other hand, are demonstrated by the small gains produced by large replacements with "vegetable albumin flour" (consisting largely of gliadin) or brewers' grains (consisting largely of residues of corn and barley proteins). Our specimens of distillers' grains (largely composed of corn protein) have shown no superiority whatever over the corn gluten itself, for the nutrition during growth.

For purposes of more strict comparison, therefore, we have selected from our numerous experiments those records in which the

food intakes during a given length of time were fairly uniform. According to our curve of normal growth a male rat weighing 60 gm. should gain 50 gm. in about 3 weeks. From the food intake data of a number of rats which have made this amount of growth in the specified time it appears that they required, on an average, 150 gm. of our foods. Taking this figure as a standard for the 3 weeks' gain indicated, and eliminating records in which the food intake showed a deviation of more than ±10 per cent, we obtain the data give in Table II.

On a *less adequate* protein mixture of the same calorific value a 60 gm. rat may require 6, 9, or even 12 weeks to gain 50 gm. in weight. From accumulated data concerning the food intake of growing rats we have estimated that the approximate amounts of food necessary to make these *slower* gains referred to are about 260, 385, and 500 gm. of food respectively. Tables III, IV, and V show the gains in weight of rats for which the food intake varied not more than ±10 per cent from these average figures. In a few protocols in these tables figures for food intake showing a deviation of somewhat more than ±10 per cent from the accepted standard have been introduced. Although they are not so strictly comparable with the rest of the records here published, they nevertheless have a comparative significance in that they represent either a gain, normal for the particular group under discussion, on a decidedly smaller food intake, or a failure to make the expected gain even when decidedly larger quantities of food were consumed.

An inspection of these tables shows that lactalbumin is by far the most efficient supplement to corn gluten of any that we have tried. When as little as one-quarter of the corn gluten is replaced by lactalbumin a rat is able to make growth at a perfectly normal rate. (Table II.) The only other supplements among those mentioned which are equally efficient in small replacements are the commercial "milkalbumin" and the soy bean flour—both effective in proportions of approximately two-thirds corn gluten to one-third of the better protein. When even about one-half of the protein is furnished as casein or edestin the otherwise similar food mixture is incapable of promoting normal growth, with food intakes of this standard magnitude.

TABLE II.

Growth of 60 Gm. Rats in 3 Weeks. The Total Food Intake in All These Trials Was Comparable and Ranged Approximately from 135 to 165 Gm. (150 ± 10 Per Cent).

Experiments with rations containing approximately 16 per cent of protein.

Protein concentrates and proteins used in the food.	Concentration of protein in food.		Rat.	Total food intake.	Body weight gained.*
	per cent			*gm.*	*gm.*
Corn gluten	11.4	15.1	1424♂	157	56
Lactalbumin	3.7				
"			1445♂	150	54
"			1432♂	159	49
Corn gluten	10.2	16.0	2111♂	158	49
"Milkalbumin"	5.8				
"			2109♂	141	45
"			2108♀	148	40
Corn gluten	10.4	16.4	3455♂	156	51
Soy bean flour	6.0				
"			3457♂	152	46
Corn gluten	7.6	16.6	3395♂	151	52
Soy bean flour	9.0				
Corn gluten	7.6	16.6	3268♂	149	48
Cottonseed flour	9.0				
Corn gluten	7.8	16.0	3540♂	159	49
Peanut meal	8.2				
Corn gluten	7.6	15.7	1353♀	153	41
Casein	8.1				
Brewers' grains		18.1	2775♂	148	27
"Vegetable albumin flour"		14.9	2676♂	148	26

* In all cases where the expected gain of 45 to 55 gm. (50 gm. ± 10 per cent) in body weight failed to be made, the figures are printed in bold-faced type.

TABLE II—*Concluded.*

Experiments with rations containing less than 15 per cent of protein.

Protein concentrates and proteins used in the food.	Concentration of protein in food.	Rat.	Total food intake.	Body weight gained.*
	per cent		*gm.*	*gm.*
Edestin	14.0	2507♂	139	33
Casein	10.8	2623♂	156	51
"		2630♂	167	58
"Milkalbumin"	9.6	2722♂	145	41
"		2716♂	142	32
Lactalbumin	9.1	2474♂	144	52
Corn oil cake	9.1	2929♂	168	39
Cottonseed flour	9.0	3368♂	140	35
Edestin	8.4	2609♂	156	28
Casein	8.1	2595♂	138	22
Corn gluten Lactalbumin	5.2 2.5 } 7.7	2270♂	168	32
Lactalbumin	7.4	2115♂	135	30
Lactalbumin	6.6	2625♂	182	45
"		2631♂	166	40
Lactalbumin	5.0	2606♂	157	21

In the experiments involving less rapid gain in weight (Table III) the relative superiority of lactalbumin is again demonstrated; for no other protein among those already tested by addition to corn gluten in equally small proportion produced a gain of 50 gm. in 6 weeks.

The growth of Rat 3331 in Table III demonstrates the difference in nutritive value between the proteins of the embryo and those of the endosperm in the corn kernel; for when about one-fourth of the protein was furnished in the form of the "corn oil cake" the rat was able to make twice as much gain in 6 weeks as

TABLE III.

Growth of 60 Gm. Rats in 6 Weeks. The Total Food Intake in All These Trials Was Comparable and Ranged Approximately from 235 to 285 Gm. (260 Gm. ± 10 Per Cent).

Experiments with rations containing approximately 16 per cent of protein.

Protein concentrates and proteins used in the food.	Concentration of protein in food.		Rat.	Total food intake.	Body weight gained.*
	per cent			*gm.*	*gm.*
Corn gluten................	13.0	14.9	3330♂	248	49
Lactalbumin................	1.9				
Corn gluten................	12.8	14.9	3281♀	267	49
Lactalbumin................	2.1				
"			3276♂	266	48
Corn gluten................	12.7	15.2	3280♂	257	66
Lactalbumin................	2.5				
Corn gluten................	13.0	15.7	3527♂	266	51
Casein................	2.7				
Corn gluten................	12.2	15.4	3324♂	236	37
Beef tissue................	3.2				
Corn gluten................	11.4	15.8	2045♂	258	74
"Milkalbumin"................	4.4				
"			2032♂	272	60
"			2048♀	238	56
Corn gluten................	11.7	15.8	3423♂	285	65
Casein................	4.1				
"			3433♂	278	62
"			3285♂	235	55
Corn gluten................	11.4	15.8	3367♂	284	58
Cottonseed flour................	4.4				
"			3369♂	251	51
"			3429♂	263	44
Corn gluten................	11.2	15.8	3331♂	284	49
Corn oil cake................	4.6				
"			3339♂	284	41
Corn gluten................	11.4	15.4	3325♂	260	44
Beef tissue................	4.0				

TABLE III—*Continued.*

Protein concentrates and proteins used in the food.	Concentration of protein in food.	Rat.	Total food intake.	Body weight gained.*
	per cent		*gm.*	*gm.*
Corn gluten	11.7 } 15.3	3390♂	309	43
Cottonseed protein	3.6 }			
"		3394♂	330	43
Corn gluten	11.7 } 15.9	3296♂	289	41
Edestin	4.2 }			
Corn gluten	10.4 } 15.8	3391♂	270±	59
Beef tissue	5.4 }			
"		3399♂	245	52
Corn gluten	10.1 } 16.1	3290♂	255	46
Cottonseed flour	6.0 }			
"		3314♂	267	46
Corn gluten	10.4 } 16.3	2815♀	245	31
Brewers' grains	5.9 }			
Corn gluten	10.4 } 15.2	3393♂	240±	39
Cottonseed protein	4.8 }			
"		3394♂	295±	44
Corn gluten	7.8 } 15.0	3576♂	268	54
Cottonseed protein	7.2 }			
Corn gluten	7.8 } 16.2	3286♂	242	48
Edestin	8.4 }			
Corn gluten	7.8 } 15.7	3486♂	279	53
Pea meal	7.9 }			
Corn gluten	7.6 } 15.1	3263♀	237	26
"Vegetable albumin flour"	7.5 }			
Brewers' grains	18.1	2791♂	277	58
"		2787♂	270	54
"Vegetable albumin flour"	14.9	2769♂	283	40
"		2764♂	266	38
"		2715♂	261	38

TABLE III—*Concluded.*

Experiments with rations containing less than 15 per cent of protein.				
Protein concentrates and proteins used in food.	Concentration of protein in food.	Rat.	Total food intake.	Body weight gained.*
	per cent		*gm.*	*gm.*
Edestin	11.2	2176♂	247	46
"		2829♂	238	45
Casein	10.8	2117♂	249	58
"		2830♂	242	54
"		2828♂	240	52
"		2831♂	248	43
Lactalbumin	9.9	2786♂	226	58
"		2782♂	233	52
"		2772♂	238	49
Lactalbumin	9.1	2478♂	257	54
"		2931♂	284	63
Corn oil cake	9.1	2937♂	289	45
Cottonseed flour	9.0	3350♂	288	57
Soy bean flour	9.0	3401♂	276	48
"		3404♂	268	42
Edestin	8.4	2598♂	266	27
"		2110♂	240	25
Casein	8.1	2595♂	278	40
"		2051♂	246	32
Lactalbumin	8.0	3192♂	252	55
"		3194♂	238	54
Beef tissue	8.0	2914♂	282	51
"Vegetable albumin flour"	7.5	2811♂	297	24
Lactalbumin	7.4	2210♂	282	62
"		2207♂	276	48
Lactalbumin	5.0	2596♂	285	44

* In all cases where the expected gain of 45 to 55 gm. (50 gm. ± 10 per cent) in body weight failed to be made, the figures are printed in bold-

TABLE IV.

Growth of 60 Gm. Rats in 9 Weeks. The Total Food Intake in All These Trials Was Comparable and Ranged Approximately from 345 to 425 Gm. (385 Gm. ± 10 Per Cent).

Experiments with rations containing approximately 16 per cent of protein.

Protein concentrates and proteins used in the food.	Concentration of protein in food.		Rat.	Total food intake.	Body weight gained.*
	per cent			gm.	gm.
Corn gluten	13.0	14.9	3425♂	362	39
Lactalbumin	1.9				
Corn gluten	13.0	15.7	3517♂	387±	61
Casein	2.7				
"			3575♂	395±	50
Corn gluten	12.2	15.4	3324♂	380	55
Beef tissue	3.2				
Corn gluten	12.5	16.1	3522♂	353	48
Cottonseed flour	3.6				
Corn gluten	11.4	15.9	3443♂	369	65
Soy bean flour	4.5				
"			3458♂	348	50
Corn gluten	11.7	15.7	3405♂	374±	64
Beef tissue	4.0				
"			3323♂	350	60
"			3325♂	406	60
Corn gluten	7.6	16.7	3332♂	362±	46
Brewers' grains	9.1				
Corn gluten	7.8	15.0	3518♂	344	48
Cottonseed protein	7.2				
Corn gluten	7.8	16.2	3438♂	323	46
Edestin	8.4				
"Vegetable albumin flour"		14.9	2715♂	390	48

TABLE IV—*Concluded.*

Experiments with rations containing less than 15 per cent of protein.				
Protein concentrates and proteins used in the food.	Concentration of protein in food.	Rat.	Total food intake.	Body weight gained.*
	per cent		*gm.*	*gm.*
Edestin	14.0	2511♂	400	59
Brewers' grains	13.3	2801♂	411	51
Edestin	11.2	2120♂	354	64
"		2176♂	399	72
Edestin	8.0	3212♂	385	51
"		3210♂	385	48
Casein	8.1	2051♂	364	45
"Vegetable albumin flour"	7.5	2811♂	486	43
"		2813♂	442	34
Lactalbumin	7.4	2123♂	318	70
Cottonseed flour	6.1	3420♂	422	50
"		3415♂	402	44
Edestin	5.6	2608♂	469	38
"		2616♂	427	33
Casein	5.4	2509♂	385	32
Corn gluten	3.5 } 5.2	2486♂	422	42
Lactalbumin	1.7 }			
"		2487♂	440	38
Lactalbumin	3.7	2044♂	350	45
"		2049♀	385	45
Lactalbumin	2.1	2203♂	367	18

* In all cases where the expected gain of 45 to 55 gm. (50 gm. ± 10 per cent) in body weight failed to be made, the figures are printed in bold-faced type.

TABLE V.

Growth of 60 Gm. Rats in 12 Weeks. The Total Food Intake in All These Trials Was Comparable and Ranged from Approximately 450 to 550 Gm. (500 Gm. ± 10 Per Cent).

Protein concentrates and proteins used in the food.	Concentration of protein in food.	Rat.	Total food intake.	Body weight gained.*
Experiments with rations containing approximately 16 per cent of protein.				
	per cent		*gm.*	*gm.*
Corn gluten	15.3	1350♂	492	48
"		1347♂	475	**34**
Corn gluten	13.0 } 14.9	3428♂	399	47
Lactalbumin	1.9			
"		3340♂	433	48
Corn gluten	11.7 } 15.8	3292♂	468	50
Casein	4.1			
Corn gluten	11.7 } 15.9	3289♂	510	53
Edestin	4.2			
Corn gluten	11.4 } 15.8	3435♂	450±	49
Cottonseed flour	4.4			
Corn gluten	7.6 } 16.7	3332♂	480±	58
Brewers' grains	9.1			
Corn gluten	5.4 } 15.9	3417♂	449	50
"Vegetable albumin flour"	10.5			
Experiments with rations containing less than 15 per cent of protein.				
Edestin	8.4	2110♀	494	62
Casein	5.4	2509♂	550	50
Edestin	4.2	2114♀	485	**33**
Lactalbumin	3.7	2044♂	479	55
"		2049♀	509	50
Lactalbumin	2.1	2203♂	499	**29**
Lactalbumin	1.9	2180♂	450	**—8**

* In all cases where the expected gain of 45 to 55 gm. (50 gm. ± 10 per cent) in body weight failed to be made, the figures are printed in bold-faced type.

when all of the protein came from the endosperm. (*Rats 1350* and 1347, Table I.) McCollum[8] has demonstrated a similarly high efficiency in promoting growth for the proteins of the wheat *embryo*.

These statistics also demonstrate the pronounced efficiency of the proteins of both the cottonseed and the soy bean as supplements to corn gluten, compared with the relative inefficiency of the proteins in brewers' grains, and "vegetable albumin flour;" for the replacement of about one-fifth or one-fourth of the corn gluten protein with the protein of the cottonseed flour or soy bean flour respectively enables a rat to make more rapid growth than substitutions of one-half or even two-thirds of the total protein by the proteins of brewers' grains and "vegetable albumin flour." (Tables IV and V.)

That the small addenda of these "better" proteins actually *supplement* the corn gluten, instead of themselves furnishing all of the protein available for growth, is shown by a comparison of the growth of rats on mixtures containing small amounts of these proteins with that of rats on equivalent or even larger quantities of the same proteins alone, *i.e.*, without corn gluten. Thus Rat 3330 (Table III) on a mixture of seven-eighths corn gluten and one-eighth lactalbumin gained 49 gm. in 6 weeks, whereas Rat 2203 (Table V) on a calorifically comparable food containing practically the same amount of lactalbumin alone—2.1 per cent—gained only 29 gm. in 12 weeks. Furthermore, Rat 3527 (Table III) on a mixture of about five-sixths corn gluten and one-sixth casein gained 51 gm. in 6 weeks, while Rat 2595 on a food containing 8.1 per cent of casein (three times as much of this protein as Rat 3527 had) gained only 40 gm. in the same time, the total food intakes of the two rats being almost identical.

A careful study of the tables presented will show numerous other instances of the efficiency of combinations of proteins in proportions in which either one alone fails to produce comparable growth. A few of these contrasts are selected for direct comparison.

Since the non-protein components of the ration used when mixed with suitable protein have been proved adequate for the

[8] McCollum, E. V., Simmonds, N., and Pitz, W., *J. Biol. Chem.*, 1916, xxv, 105.

TABLE VI.

Rat	Protein concentrates and proteins used in the food.	Concentration of protein in food.	Body weight gained.	Time.
		per cent	*gm.*	*wks.*
1424♂	Corn gluten Lactalbumin	11.4 3.7	56	3
2044♂	Lactalbumin	3.7	55	12
3527♂	Corn gluten Casein	13.0 2.7	51	6
2509♂	Casein	5.4	50	12
3289♂	Corn gluten Edestin	11.7 4.2	53	12
2114♀	Edestin	4.2	33	12
3290♂	Corn gluten Cottonseed flour	10.1 6.0	46	6
3522♂	Corn gluten Cottonseed flour	12.5 3.6	48	9
3420♂	Cottonseed flour	6.0	50	9
3455♂	Corn gluten Soy bean flour	10.4 6.0	51	3
3443♂	Corn gluten Soy bean flour	12.3 4.5	38	6
3401♂	Soy bean flour	9.0	48	6
3331♂	Corn gluten Corn oil cake	11.2 4.5	49	6
3410♂	Corn oil cake	4.5	10	6

growth of rats, any nutritive deficiency in the diets under discussion may be referred to the amino-acid make-up of the proteins employed. Previous experiments have shown that the inefficiency of zein which forms 31.3 per cent of the protein of corn gluten is due to a lack of lysine and tryptophane among its products of hydrolysis.[4] That the inefficiency of corn gluten is also due to this cause is demonstrated by recent experiments not heretofore published (Chart I). The addition of either amino-acid by itself failed to promote any noticeable increase in the rate of growth. Bearing this fact in mind, it is easy to understand why the brewers' and distillers' grains and the "vegetable albumin flour" have

TABLE VIII.

Rat.	Concentration of protein in the food.				Body weight gained.	Food intake.	Time.
		per cent		per cent	gm.	gm.	wks
3256 ♀	Corn gluten	7.6 +	distillers' grains	8.0	−1	155	4
	"	"	cottonseed flour	9.0	+34	168	4
3257 ♀	"	"	distillers' grains	8.0	10	162	4
	"	"	soy bean flour	9.0	23	138	4
3263 ♀	"	"	"vegetable albumin flour"	7.5	26	276	7
	"	"	cottonseed flour	9.0	33	268	7
3264 ♀	"	"	"vegetable albumin flour"	7.5	17	246	7
	"	"	soy bean flour	9.0	32	243	7

food intakes, although during the second period the rats had become appreciably larger than in the first period, and might reasonably be expected to require more food.

SUMMARY.

Data regarding the growth of rats are presented to show the relative nutritive values of a number of proteins and protein "concentrates" when used to supplement corn gluten in an otherwise adequate ration. The products studied included casein, lactalbumin, edestin, cottonseed protein, cottonseed flour, soy bean flour, "milkalbumin," beef tissue, fish meat meal, corn oil cake, "vegetable albumin flour," brewers' grains, distillers' grains, pea meal, and peanut meal. Such food mixtures of approximately the same nitrogen and calorific content vary greatly in their efficiency for promoting growth, in accordance with both the character and the proportion of the protein supplement employed.

The efficiency of these supplements presumably depends essentially upon their relative content of lysine and tryptophane; for the addition of these amino-acids, either as such, or in the form of proteins yielding them, renders corn gluten suitable for growth.

Of the various proteins employed to supplement the inefficient corn gluten, lactalbumin is by far the most effective. Satis-

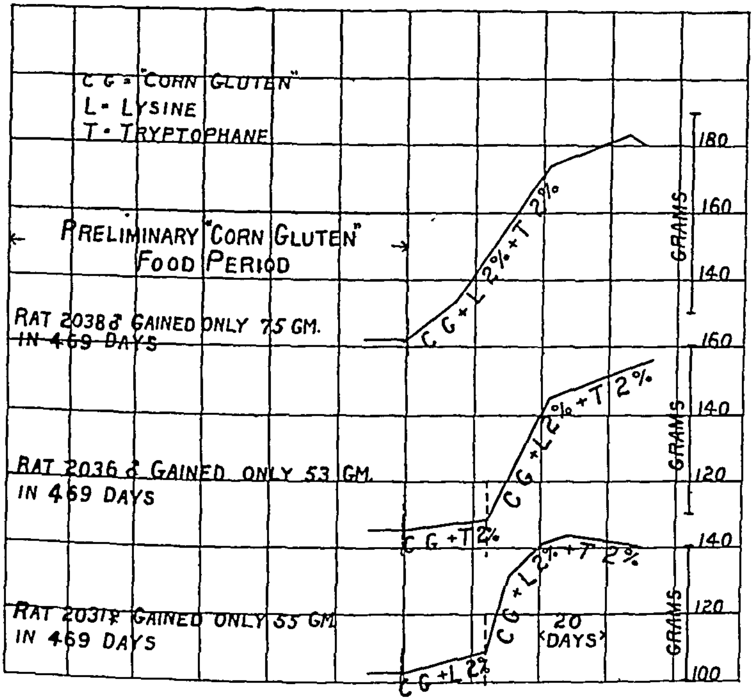

CHART I. Showing the resumption of growth when the amino-acids lysine and tryptophane were added to a diet containing corn gluten as the source of nitrogen. Neither of these amino-acids alone sufficed to induce an increased rate of growth when added to the corn gluten ration deficient in both of them. The foods consisted of:

	Preliminary stunting periods.	Subsequent periods.
	per cent	*per cent*
Corn gluten	25–38	38.00
Amino-acids	0	0.36–1.72
"Protein-free milk"	28	28
Starch	2–15	1.64–1.28
Butter fat	3–18	18
Lard	14–29	14

factory growth is produced with smaller quantities of this protein than of any of the others recorded. The samples of brewers' grains, distillers' grains, and "vegetable albumin flour" used were the least efficient supplements tested, presumably because of their low content of lysine.

It is evident that the small additions of the more efficient proteins actually *supplement* the corn gluten instead of themselves furnishing all of the protein used for growth, because equivalent amounts of these proteins alone in a similar ration are incapable of inducing a comparable degree of growth. Small amounts of a superior protein are often just as efficient for growth as larger amounts of a less adequate protein.

The plan of feeding and comparison here described affords a practical method of studying accurately on a small scale the comparative nutrient value of the nitrogenous components of commercial feedingstuffs.

CHOLESTEROL IN HUMAN BLOOD UNDER PATHOLOGICAL CONDITIONS.

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During the past 10 years a considerable number of observations have been published concerning the cholesterol content of the blood in various pathological conditions. A hypercholesterolemia has been noted in nephritis, diabetes, lues, pregnancy, cholelithiasis, obstructive jaundice, obesity, alcoholism, starvation, typhoid fever, many skin diseases, in malignant disease, in cachexias of various origins, and after narcosis. Cholesterol has been found in subnormal quantities in the blood of persons suffering from anemia, diseases of the liver (without jaundice), in certain cases of nephritis, in many acute infections, and in a variety of conditions "where the vitality is low."[1]

The number and variety of conditions in which an abnormal cholesterol content of the blood is reported are unconvincing, the more so as on examination of the literature it is apparent that the analytical methods employed by some of the frequently quoted clinical investigators in this field leave much to be desired. In view of the rapidly increasing importance of blood analysis, both to the biological chemist and to the clinician, it has seemed worth while, in spite of the relatively large number of papers to be found in the literature dealing with blood cholesterol in pathological conditions, to publish the following series of observations in which determinations of cholesterol have been made on the blood of persons suffering from the more common chronic and acute diseases.

The results reported have in every case been obtained by Bloor's modification[2] of the Autenrieth-Funk colorimetric method

[1] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.

[2] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

a procedure which has been selected because of the small quantity of blood required, the relative ease of manipulation, and the freedom from procedures which might lead to decomposition of cholesterol.

Bloor[1] has published a series of cholesterol figures for the blood of normal men and women as determined by the above method. In a series of nine normal women he observed a maximum blood cholesterol of 0.240 per cent and a minimum of 0.210 per cent. In a series of fourteen normal men the maximum is 0.250 per cent, the minimum 0.190 per cent. These figures are considerably higher than those ordinarily given in the literature, a fact probably due to the more complete extraction secured by the method of analysis employed.

In view of the relatively small number of normal bloods examined by Bloor it has seemed worth while to obtain a few more figures on normal persons. The bloods used in this series were obtained from the nurses and physicians in the hospital, all relatively young people, and from a few middle aged and old men and women who were convalescents, on the surgical services, from fractures and amputations.

TABLE I.

Blood Cholesterol in Normal Persons.

No.	Sex.	Cholesterol per 100 cc. of blood.	No.	Sex.	Cholesterol per 100 cc. of blood.
		mg.			*mg.*
C235	♀	167	C255	♂	255
C236	♀	179	B140	♂	250
B3	♀	200	B143	♂	198
B4	♀	194	B19	♂	240
B5	♀	225	42	♂	192
B6	♀	190	45	♂	250
C196	♂	223	46	♂	228
C230	♂	211	59	♂	208
C229	♂	210	133	♂	223
C251	♂	200	136	♂	249

From this series it is apparent that cholesterol values on normal blood may vary from 167 to 255 mg. per 100 cc. of blood, a variation somewhat wider than that given by Bloor.

In connection with the statements to be found in the literature regarding the hypercholesterolemia found in the obese, it is interesting to note that the lowest figure in Table I (167 mg.) was obtained on the blood of a woman weighing 200 pounds, while the next lowest figure (179 mg.) was obtained on the blood of a woman weighing 260 pounds.

Before beginning the collections of blood it seemed desirable to determine whether the ingestion of food, particularly food rich in lipoids, has any immediate effect on the cholesterol content of the blood. It has long been known that prolonged cholesterol feeding produces in animals an increase in the cholesterol content of the blood.[3] No statements are, however, to be found in the literature concerning the effect, if any, produced by a single lipoid-rich meal.

In order to determine this point three men were selected (convalescent fracture cases) who had sufficiently large appetites to eat a breakfast consisting of four eggs, 200 gm. of mutton chop, 50 gm. of bacon, ½ pint of cream, bread, butter, and coffee. Blood was taken just before breakfast, at which time the patients had been fasting for 15 hours, and again 3 hours after the meal.

	Cholesterol per 100 cc. of blood.	*mg.*
L.	Fasting	250
	3 hrs. after breakfast	250
H.	Fasting	185
	3 hrs. after breakfast	181
B.	Fasting	210
	3 hrs. after breakfast	212

As will be seen from the above results the ingestion of lipoid-rich food has no immediate effect on the cholesterol content of the blood, and I have therefore felt justified in taking my samples of blood at any convenient hour, although they were in a large majority of cases taken about 4 hours after the midday meal.

Blood Cholesterol in Nephritis.

On account of the fundamental part in the formation of arteriosclerotic lesions which has been ascribed to cholesterol by some

[3] Grigaut, A., and L'Huillier, A., *Compt. rend. Soc. biol.*, 1912, lxxiii, 304. Gardner, J. A., and Lander, P. E., *Biochem. J.*, 1913, vii, 576. Luden, G., *J. Biol. Chem.*, 1916, xxvii, 273.

TABLE II.

Blood Cholesterol in Nephritis.

No.	Sex.	Age.	Diagnosis.	Per 100 cc. of blood.	
				Non-protein nitrogen.	Cholesterol.
		yrs.		*mg.*	*mg*
51	♂	42	Chronic nephritis. Hypertension.	32	250
48	♂	35	" " Uremia. Died 10 days later.	69	257
50	♂	42	Chronic nephritis.	43	257
49	♂	38	Nephritis. Acute exacerbation following infection of hand. Died 18 hrs. after blood was taken.	187	250
205	♂		Nephritis. Hypertension. Arteriosclerosis. Much edema.	36	238
C267	♂		Nephritis. Hypertension. Arteriosclerosis.	88	213
C245	♂	53	Chronic nephritis. Hypertension. Arteriosclerosis. Retinitis. Phthalein test 20 per cent in 2 hrs.	51	292
C199	♀	45	Chronic nephritis. Arterial walls much thickened and very tortuous. Retinitis. Hypertension. Phthalein test 15 per cent in 2 hrs.	55	265
53	♂		Chronic nephritis. Hypertension.	31	245
44	♂	23	Acute "	100	232
47	♂	44	Chronic "	42	228
62	♀	60	" " Marked arteriosclerosis.	54	217
136	♀	54	Chronic nephritis.	33	238
153	♂	48	Chronic interstitial nephritis. Hypertension. Uremia. Phthalein test 1 per cent in 2 hrs. Died 2 days after blood was taken.	238	208
220	♂		Chronic nephritis. Marked arteriosclerosis. Hypertension.	48	211
129	♂		Chronic nephritis.	76	206
C231	♂	40	" " Hypertension. Albuminuric retinitis. Phthalein test 2 per cent in 2 hrs. Died 2 days later.	234	201
C173	♂	80	Chronic nephritis. Marked arteriosclerosis. Phthalein test 2 per cent in 2 hrs. Died 4 days after blood was taken.	54	211
C200	♂	19	Acute nephritis. Slight edema. Phthalein test 30 per cent in 2 hrs.	45	245

TABLE II—*Continued.*

No.	Sex.	Age.	Diagnosis.	Per 100 cc. of blood. Non-protein nitrogen.	Cholesterol.
		yrs.		*mg.*	*mg.*
226	♂	52	Chronic nephritis. Marked arteriosclerosis. Slight edema.	81	208
C254	♂	40	Chronic nephritis. Phthalein test 45 per cent in 2 hrs.	71	216
54	♂		Chronic nephritis. Slight arteriosclerosis. Phthalein test 53 per cent in 2 hrs.	37	212
126	♀	16	Acute nephritis. Phthalein test 14 per cent in 2 hrs. Much edema.	75	208
225	♂	54	Chronic nephritis. Moderate edema. Phthalein test 25 per cent in 2 hrs.	71	199
118	♀	23	Chronic nephritis. Much edema.	39	166
112	♂	39	" " Very slight edema. Moderate arteriosclerotic changes. Phthalein test 5 per cent in 2 hrs.	125	178
113	♂	45	Chronic nephritis. Phthalein test 40 per cent in 2 hrs.	55	178
139	♂	65	Chronic nephritis. Moderate edema.	45	181
130	♂	53	" " Chronic plumbism. Marked arteriosclerotic changes.	65	169
206	♀	33	Chronic nephritis. Much edema.	36	165
56	♀	62	" " Moderate arteriosclerotic changes.	62	146
129		52	Chronic nephritis. Moderate edema.	76	206
140			Same patient as above after 3 weeks' treatment.	40	158
44		23	Acute nephritis. Moderate edema. Phthalein test 15 per cent in 2 hrs.	100	232
B82			Same patient 28 days later.	50	192
B99			" " 42 " "	41	245
C304			" " 63 " " Phthalein test 50 per cent in 2 hrs.	38	225
C221		40	Chronic nephritis. Hypertension. Albuminuric retinitis. Phthalein test 2 per cent in 2 hrs.	111	181
C231			Same patient as above 6 days later. Blood taken while in uremic convulsion. Died in 36 hrs.	234	201

TABLE II—*Concluded.*

No.	Sex.	Age.	Diagnosis.	Per 100 cc. of blood. Non-protein nitrogen.	Per 100 cc. of blood. Cholesterol.
		yrs.		*mg.*	*mg.*
C246		38	Chronic nephritis. Arteriosclerosis. Uremia. Phthalein test 1 per cent in 2 hrs.	89	231
C260			Same patient as above 4 days later; growing steadily worse.	192	190
C265			Same patient 6 days later. Died 24 hrs. after this blood sample was taken.	256	194
C307			Same patient, blood taken 1 hr. before death.	298	187
C253		20	Acute nephritis. No hypertension. No arteriosclerotic changes. Albuminuric retinitis. Phthalein test 10 per cent in 2 hrs. Very severe edema.	42	475
C302			Same patient 4 weeks later. No improvement.	40	606

TABLE III.

Blood Cholesterol in Cardiorenal Disease.

No.	Age.	Diagnosis.	Per 100 cc. of blood. Non-protein nitrogen.	Per 100 cc. of blood. Cholesterol.
	yrs.		*mg.*	*mg.*
43	65	Chronic nephritis. Mitral regurgitation.	50	250
55	48	" " " "		181
57	59	Cardiorenal disease. Marked arteriosclerosis.		178
58	56	Chronic nephritis. Mitral stenosis and regurgitation.		203
60	66	Chronic nephritis. Chronic endocarditis.		166
61	55	Cardiorenal disease.		166
134	48	" "	44	160
137	67	" " Marked edema. Arterial walls palpable.	48	178
138	51	Cardiorenal disease. Marked edema. Arterial walls show slight sclerosis. Phthalein test 16 per cent in 2 hrs.	39	227
141	68	Cardiorenal disease.	41	200
152	70	Chronic nephritis. Mitral disease.	55	238
C222	58	Chronic nephritis. Mitral regurgitation. Arteriosclerosis. Cardiac hypertrophy. Phthalein test 15 per cent in 2 hrs.	65	174

TABLE IV.
Blood Cholesterol in Cardiac Disease.

No.	Age.	Diagnosis.	Per 100 cc. of blood. Non-protein nitrogen.	Cholesterol.
	yrs.		*mg.*	*mg.*
155		Mitral regurgitation. Decompensation.	38	279
95		Aortic disease. Marked decompensation.	37	300
97		Mitral stenosis. Decompensation.	37	193
98		Mitral regurgitation. Decompensation. Ascites.	37	217
99		Chronic endocarditis. Decompensation.	45	250
100		" "		
101		" "		176
102		Aortic disease. Decompensation.		225
72		Mitral stenosis and regurgitation.	30	186
218		" " " "		176
C233	65	Aortic and mitral disease. Arteriosclerosis. Angina pectoris.	40	268
C252	58	Valvular disease. Decompensation.		240
C195	50	Mitral regurgitation. Decompensation.	43	222
C171	28	Aortic and mitral disease.		141
C268	50	Mitral regurgitation. Decompensation.	38	250

writers, much attention has been given to the collection of quantitative data concerning the occurrence of this body in nephritic blood.

In general, the opinion has been that a hypercholesterolemia occurs with great frequency in patients with chronic Bright's disease, cardiorenal disease, arteriosclerosis, and related conditions.[4] According to these investigators, however, this hypercholesterolemia is not a retention phenomenon, as shown by the fact that in patients showing a high content of urea in the blood, the cholesterol figures tend to fall, and may even become subnormal (Schmidt), while in three cases it was noted that a rise in the non-protein nitrogen of the blood was coincident with a fall in the cholesterol content (Widal[4]).

[4] Chauffard, A., Laroche, G., and Grigaut, A., *Compt. rend. Soc. biol.*, 1911, lxx, 108. Widal, F., Weill, A., and Laudat, M., *Semaine méd.*, 1912, xxxii, 529. Schmidt, H. B., *Arch. Int. Med.*, 1914, xiii, 127. Henes, E., *Deutsch. Arch. klin. Med.*, 1913, cxi, 122.

The above results do not confirm the findings of the earlier investigators concerning the high cholesterol content of nephritic blood. In one case only was a hypercholesterolemia observed. It should be pointed out, however, that these patients were all on a "nephritic diet" containing no meat, and few eggs or other foodstuffs of a high lipoid content. In the four cases in which a series of determinations were made at intervals during the progress of the disease there is no parallelism to be noted between the non-protein nitrogen and the cholesterol content of the blood.

Blood Cholesterol in Syphilis.

It has been reported that blood cholesterol is present in increased quantities in the blood of syphilitics[5] and the suggestion has even been made that this increased lipoid content may account in part at least for the "Wassermann reaction" given by luetic sera. On the other hand, Stein[6] has reported the cholesterol content of syphilitic blood to be normal in primary cases and low in secondary infections.

TABLE V.

Blood Cholesterol in Syphilis.

No.	Sex.	Age.	Diagnosis.	Cholesterol per 100 cc. of blood.
		yrs.		*mg.*
150	♂		Gastric syphilis.	250
14	♂		Enlarged liver. Wassermann strongly positive.	175
C42	♂		Syphilis.	173
C43	♂	33	Early tertiary lesion.	135
C248	♀	22	" primary "	166
C250	♀	15	Congenital syphilis.	170
C252	♀	16	" "	250
C220	♂	39	Late secondary lesion.	150
C237	♂	56	Syphilis. Peptic ulcer.	151
C239	♂	38	Tabes. Gastric ulcer.	238
194	♀		Syphilitic ulcer of the leg.	167
201	♀		" "	238
C264	♂		Gastric syphilis.	226
C237	♂		Syphilis of central nervous system.	158

[5] Klein, W., and Dinkin, L., *Z. physiol. Chem.*, 1914, xcii, 302.

[6] Stein, G., *Z. ges. exp. Med.*, 1914, iii, 309.

In the fourteen specimens of luetic blood examined no increase was observed in any case, in fact many gave extremely low cholesterol values.

Blood Cholesterol in Pregnancy.

An increased cholesterol content of the blood has been described in pregnant women.[7] In the case of twelve women in early pregnancy whose blood was examined no increase in cholesterol was noted.

TABLE VI.

Blood Cholesterol in Pregnancy.

No.	Diagnosis.	Cholesterol per 100 cc. of blood.
		mg.
C202	Normal pregnancy.	183
C203	" "	236
C204	" "	260
C205	" "	223
C206	" "	223
207	" "	221
224	" "	283
225	" "	224
201	Probable toxemia.	256
226	Normal pregnancy.	272
241	Persistent vomiting.	208
262	" "	224

The next series of observations would seem to indicate that in diabetes a hypercholesterolemia is of rare occurrence. Taken as a whole there seems to be no definite relation as far as can be judged by the analytical data presented either between the degree of acidosis and the blood cholesterol or between the blood sugar and cholesterol.[8]

[7] Herrmann, E., and Neumann, J., *Biochem. Z.*, 1912, xliii, 47. Neumann and Herrmann, *Wien. klin. Woch.*, 1911, xxiv, 411. Herrmann and Neumann, *ibid.*, 1912, xxv, 1557. Albrecht, H., and Weltmann, O., *ibid.*, 1911, xxiv, 483. Landau, M., *Deutsch. med. Woch.*, 1913, xxxix, 546.

[8] All samples of diabetic blood were taken before breakfast in order to avoid the complication of an alimentary glycosuria. The blood sugar determinations were made by Myers' modification of the Lewis-Benedict method.

TABLE VII.

Blood Cholesterol in Diabetes.

No.	Sex.	Age.	Urinary sugar.	Diacetic acid.	Blood sugar.	Cholesterol per 100 cc. of blood.
		yrs.	*per cent*		*per cent*	*mg.*
87	♂		0.8	+	0.15	186
88	♀		2.2	+	0.16	277
89	♀		Trace.	+	0.14	235
90	♂		6.2	+	0.16	292
91	♂		5.4	++	0.28	312
92	♂		1.4	+	0.13	225
93	♂		0	0	0.10	185
94	♀		Trace.	+	0.150	207
214	♂		2.0	++	0.25	332
216	♀		2.2	+	0.18	250
125	♀		0	0	0.097	172
127	♀		0	0	0.093	219
132	♂		0	0	0.125	166
120	♀		0	0	0.111	150
C216b	♀		2.0	+	0.18	274
C217	♀		0.5	+	0.164	256
C49	♂	50	0.79	+	0.19	166
C175	♀	44	1.0	++	0.19	250
C234	♂	35	2.8	++	0.28	300
C298	♀	42	2.2	+	0.31	183
C297	♀	52	0	0	0.14	314
C288	♂	44	0	0	0.17	245
C287	♀	28	2	0	0.17	189
C286	♂	33	0	0	0.16	367
C299	♂		1.4	+	0.16	205

Blood Cholesterol in Acute Infections.

But little work has been published regarding the effect of acute infections on the cholesterol content of the blood. Chauffard, Richet, and Grigaut[9] have found that in typhoid fever high cholesterol values are obtained in the late stages of the disease, whereas in pulmonary tuberculosis a subnormal amount is present.

The results presented on cases of acute infections show in the severe cases a marked diminution of the cholesterol content of the blood, which rises to normal values during convalescence. In a few very mild cases of typhoid no hypercholesterolemia was noted.

[9] Chauffard, Laroche, and Grigaut, *Compt. rend. Soc. biol.*, 1911, lxx, 70; Chauffard, A., Richet, C., and Grigaut, A., *ibid.*, 1911, lxx, 276.

TABLE VIII.

Blood Cholesterol in Acute Infections.

No.	Sex.	Diagnosis.	Cholesterol per 100 cc. of blood.
			mg
140	♀	Typhoid, 2nd week. Severe case.	173
b2		Same patient 7 weeks later, convalescent.	205
147	♂	Typhoid, 2nd week. Mild case.	228
		Same patient. Late convalescence, 6 weeks later.	226
	♂	Typhoid, 3rd week. Mild case.	205
		Same patient 5 weeks later, convalescent.	222
	♂	Typhoid 3rd week. Severe case. Relapse.	145
		Same patient 9 weeks later, convalescent.	168
C151	♂	Typhoid, 2nd day of normal temperature. Severe case.	150
C182		Same patient 18 days later.	303
C164	♂	Typhoid, 4th day of normal temperature. Severe case.	164
		Same patient 4 weeks later.	210
146	♂	Typhoid, 4th week. Mild case.	168
149	♂	" early convalescence.	205
C284	♂	" 2nd week.	139
C281	♂	" 2nd "	139
C192	♂	Pneumonia. Pulmonary tuberculosis.	139
C300	♂	Same case convalescent, 7 weeks later.	162
C295	♂	Bronchial pneumonia.	151
C294	♂	Lobar pneumonia. Before crisis.	141
C289	♂	" " 12 hrs. after crisis.	166
C285	♂	" " Before crisis.	129
C278	♀	Bronchial pneumonia.	171
66	♂	Acute pleurisy.	279
C283	♂	" " with effusion.	145
75	♂	Pneumonia. Before crisis.	151
C214	♀	" " "	143
C215	♂	" " "	133
C216	♂	" Crisis 6 days later.	166
70	♂	Acute rheumatic fever.	169
C263	♂	" " "	150
104	♂	Septic foot and leg. Severe case.	203

Blood Cholesterol in Diseases of the Liver (Including Cholelithiasis).

Blood cholesterol determinations in cases of gall-stone disease have always been popular and numerous attempts have been made to connect the occurrence of cholelithiasis with a hypercholesterolemia.[10] In obstruction of the common duct an increase in cholesterol has been noted,[11] while in cirrhosis of the liver subnormal quantities of cholesterol have been reported.

TABLE IX.

Blood Cholesterol in Diseases of the Liver.

No.	Sex.	Diagnosis.	Cholesterol per 100 cc. of blood.
			mg.
142	♂	Cirrhosis of liver. Ascites.	166
13	♂	" " " Alcoholic. Marked jaundice.	170
272	♀	" " " Syphilitic. " "	170
C271	♂	" " " *Alcoholic.* " "	*160*
C280	♂	" " " Slight jaundice.	205
10	♂	Gall-stones (operation).	222
11	♂	" "	227
12	♀	" "	204
	♂	" " *Slight jaundice.*	*174*
C275	♀	" "	188
C274	♂	" "	150
D66	♀	" " Marked jaundice.	200
B43	♀	" " Slight "	183
B37	♀	" " " "	192
C191	♀	Abscess of liver. Marked jaundice.	154
208	♂	Catarrhal jaundice. Marked. Much bile in urine.	146
C279	♂	Chronic disease of gall-bladder. Much bile in urine.	273

The series of liver cases is not large, but it gives no indication that hypercholesterolemia is necessarily an accessory to gall-stone disease. In the nine cases of cholelithiasis observed, the presence of stones was in every case proved by operation. In the

[10] Henes, *J. Am. Med. Assn.*, 1914, lxiii, 146. Rothschild, M. A., and Rosenthal, N., *Am. J. Med. Sc.*, 1916, clii, 394.

[11] Chauffard, Laroche, and Grigaut, *Compt. rend. Soc. biol.*, 1911, lxx, 70. Schmidt, *Arch. Int. Med.*, 1914, xiii, 125.

cases examined which showed icteric symptoms of various degrees of severity no increase of blood cholesterol was noted.

Blood Cholesterol in Malignant Disease.

A hypercholesterolemia has been described in the early stages of malignant disease[12] while in advanced tumor cases where cachexia is present the cholesterol of the blood is low. The samples of blood examined taken from persons suffering from malignant disease have, however, given in every case results within, or in a few cases below, normal limits.

TABLE X.

Blood Cholesterol in Malignant Disease.

No.	Sex.	Diagnosis.	Cholesterol per 100 cc. of blood.
			mg.
16	♂	Carcinoma of transverse colon.	250
17	♂	" " stomach.	233
18	♂	Hypernephroma of tibia and humerus.	178
19	♂	Tumor of cord.	250
20	♀	Abdominal tumor.	182
21	♀	Inoperable carcinoma of breast.	195
22	♀	Malignant disease of the liver (marked jaundice).	277
23	♀	Carcinoma of stomach.	178
24	♂	Abdominal tumor.	154
143	♂	Malignant disease with multiple metastasis.	146
40	♂	Cancer of stomach (marked anemia).	130
C261	♀	Recurrent carcinoma of breast.	201
C303	♂	Abdominal tumor.	257
C290	♂	Cancer of stomach.	238

Blood Cholesterol in Diseases of the Skin.

In a study of blood cholesterol in diseases of the skin Fischl[13] has reported that in the ordinary dermatosis not accompanied by fever, such as urticaria, eczema, mycosis fungoides, etc., the cholesterol content of the blood is high, while in conditions accompanied by fever, such as erysipelas, herpes zoster, etc., a hypercholesterolemia is found.

[12] Luden, *J. Lab. and Clin. Med.*, 1916, i, 662.

[13] Fischl, F., *Wien. klin. Woch.*, 1914, xxvii, 982.

TABLE XI.

Blood Cholesterol in Diseases of the Skin.

No.	Diagnosis.	Sex.	Cholesterol per 100 cc. of blood.	No.	Diagnosis.	Sex.	Cholesterol per 100 cc. of blood.
			mg.				mg.
156	Eczema.	♂	173	168	Psoriasis.	♀	168
157	"	♂	196	169	"	♀	165
158	"	♀	145	177	"	♀	166
159	"	♂	147	179	"	♀	230
160	"	♂	227	180	"	♀	163
171	"	♂	168	187	"	♀	250
172	"	♂	195	231	"	♀	194
175	"	♂	168	161	Prurigo.	♂	151
182	"	♂	157	162	Erythema multiforme.	♂	231
190	" Jaundice.	♂	172	163	Mycosis fungoides.	♂	164
191	"	♀	194	164	Mycosis fungoides.	♂	146
195	"	♀	175	167	Vitiligo.	♀	216
227	"	♀	200	165	Lupus erythematosus.	♀	206
229	"	♀	194	170	Tubercular lesion.	♂	233
230	"	♂	192	204	Lupus erythematosus.	♂	200
174	" seborrheic.	♀	156	183	Lichen planus.	♀	195
166	" psoriasiform.	♀	184	189	" "	♀	234
228	Acne vulgaris.	♂	195	184	Scabies.	♂	186
185	" "	♂	208	186	Sporotrichosis.	♂	208
193	" "	♀	208	188	Actinodermatitis.	♂	238
197	" "	♂	185	192	Scleroderma.	♀	208
198	" "	♂	162	200	Herpes zoster.	♂	255
172	" "	♀	208	203	Pityriasis rosea.	♀	226
178	Furunculus.	♂	201				
199	"	♂	177				
176	Urticaria.	♂	208				
181	"	♀	208				
196	"	♀	166				
202	" (chronic).	♀	189				

The results presented in Table XI indicate that, in the main, persons suffering from the ordinary types of dermatoses show essentially normal blood cholesterol values. Of seventeen cases of eczema examined, it is to be noted that three show abnormally low values. Out of a total of seven cases of psoriasis four gave cholesterol figures approaching the minimal normal values, while the same may be said of one case of prurigo, two of mycosis fun-

goides, one of acne vulgaris, and one of urticaria. The cases of eczema and of psoriasis were on restricted diets, which may in part at least account for the low values obtained. The other cases giving subnormal figures were on an unrestricted food intake but were without exception old persons who had suffered from these diseases for many years and who were physically much below par.

Blood Cholesterol in Anemia.

Many observations have been published showing that the cholesterol content of the blood in anemic conditions is invariably low. In fact this finding has been so universal that cholesterol injections have even been tried in Italy as a cure for pernicious anemia.

The observations recorded below confirm these results. It should be noted, however, that the cholesterol figures vary relatively but not absolutely with the red cell count. That the anemia as such has no relation to the hypercholesterolemia, is suggested by Case 41, a vigorous young man who had suffered a severe hemorrhage, and who showed an absolutely normal cholesterol value with a blood count of less than 2,000,000.

Three cases are included in which marked improvement in the blood picture had taken place after treatment (transfusion). In one of these although the red cell count is more than doubled the cholesterol content of the blood decreased slightly, while in two other cases with an increase of 100 per cent in the cell content of the blood the cholesterol increased in one case only a trifle more than 50 per cent and in the other less than 20 per cent.

In connection with the cases of anemia two cases of polycythemia examined are worthy of mention on account of the normal cholesterol values present.

No. 124. Pulmonary stenosis with open red cells, 9,400,000 hemoglobin 148 per cent, cholesterol 170 mg. per 100 cc. of blood.

No. 25. Enlarged liver and spleen (cause?) red cells 9,200,000, hemoglobin 130 per cent, cholesterol 180 mg. per 100 cc. of blood.

TABLE XII.

Blood Cholesterol in Anemia.

No.	Diagnosis.	Red cells per c. mm.	Hemoglobin (Sahli).	Cholesterol per 100 cc. of blood.
			per cent	*mg.*
26	Pernicious anemia.	500,000	12	135
116	" "	1,000,000	20	126
117	" "	1,400,000	30	128
111	" "	2,200,000	55	139
114	" "	1,800,000	50	154
119	" "	900,000	30	166
118	" "	1,800,000	50	172
29	" "	2,300,000	60	169
32	" "	1,700,000	30	219
36	" "	1,200,000	35	130
36a	Same patient after treatment.	2,500,000	80	210
37	Pernicious anemia.	824,000	38	150
37a	Same patient after treatment.	2,100,000	60	130
38	Pernicious anemia.	2,000,000	30	180
38a	Same patient after treatment.	3,900,000	75	210
28	Splenic anemia.	2,200,000	30	190
C273	Banti's disease.	2,700,000	36	144
39	Leukemia.			176
C190	Lymphatic leukemia (acute).	1,300,000	26	144
143	Malignant disease with multiple metastases. Secondary anemia.	2,100,000	28	146
40	Cancer of stomach. Secondary anemia.	3,000,000	50	130
33	Tapeworm. Secondary anemia.		60	147
41	Gastric ulcer accompanied by severe hemorrhage. Secondary anemia.	1,840,000	29	208

Blood Cholesterol in Diseases of the Ductless Glands.

On account of the part ascribed to the ductless glands in the regulation of lipoid metabolism I have made a few examinations of blood from persons suffering from thyroid disease and in one case of acromegaly. As will be seen from the following result, no striking deviations from the normal values were found, al-

though it is of interest to note that four of the five cases of severe Graves' disease gave low figures, while the one case of myxedema examined showed a figure a trifle above the normal. No relation is apparent between blood sugar and blood cholesterol.

TABLE XIII.

Blood Cholesterol in Diseases of the Ductless Glands.

No.	Sex.	Diagnosis.	Blood sugar.	Cholesterol per 100 cc. of blood.
			per cent	*mg.*
76	♀	Hyperthyroid, severe case.	0.10	176
212	♂	" " "	0.096	163
C291	♂	" " "	0.10	178
C276	♀	" " "	0 11	180
C270	♀	" " "	0.11	231
C168	♀	" mild case.	0.096	238
C189	♀	" " "	0.12	247
C228	♂	" " "	0.11	186
C306	♀	" " "	0.10	257
C305	♀	" " "		225
C227	♀	Myxedema.	0.12	295
B102	♀	Acromegaly.	0.11	280

SUMMARY.

Determinations of cholesterol have been made on the blood of twenty normal individuals and of two hundred and fifty-four persons suffering from a variety of the more common diseases and including twelve in pregnancy. As a result of this work the following conclusions are drawn.

1. In nephritis, cardiorenal disease, arteriosclerosis, and cardiac disease the blood cholesterol remains at normal levels both in the early stages of the disease and when the patient is practically moribund. There is no relation between the non-protein nitrogen of the blood and the cholesterol.

2. In syphilis the blood cholesterol is not increased. In some cases it is low.

3. In twelve cases of early pregnancy no increase in blood cholesterol was noted.

4. In twenty-five diabetics, five showed a slight increase in the cholesterol content of the blood. This hypercholesterolemia bore

no constant relation to the blood sugar or to the acetone bodies or sugar of the urine.

5. In the case of the acute infections, typhoid fever, pneumonia, pleurisy, and rheumatic fever were studied. In these conditions low cholesterol values were found when the patient was very ill; in convalescence normal values were reestablished.

6. In nine cases of gall-stone disease no marked increase of blood cholesterol was found. In icterus even when severe there was no increase. In cirrhosis of the liver the values were within the lower normal limits.

7. In fourteen early cases of malignant disease the cholesterol figures were normal except in one case associated with anemia in which the value was low.

8. In diseases of the skin the blood cholesterol figures obtained were within normal limits.

9. In severe primary and secondary anemias subnormal values were obtained. No definite relation was found to exist between the number of corpuscles or hemoglobin percentage and cholesterol values.

10. In severe hyperthyroidism figures were obtained within or just below the lower normal limit. In one case of myxedema a cholesterol figure slightly above the highest normal value was found.

CONCLUSIONS.

Among the pathological conditions examined hypercholesterolemia was found to exist only in diabetes and only in a relatively small number of cases of this disease. Low cholesterol values are not characteristic of any special pathological condition but are invariably found in conditions where marked prostration or cachexia exist. Cholesterol determinations in the blood are therefore at present of no value in the clinical diagnosis or prognosis of disease.

I am indebted to my assistant, Miss Anna S. Minot, for many of the determinations recorded in this paper.

ADENINE-URACIL DINUCLEOTIDE AND THE STRUCTURE OF YEAST NUCLEIC ACID.

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Recent investigations have necessitated the assumption that yeast nucleic acid contains groups of the four following mononucleotides.[1-7]

$$\begin{matrix}HO\diagdown \\ O=P-O\,.\,C_5H_8O_3\,.\,C_5H_4N_5 \\ HO\diagup\end{matrix}\quad \text{adenine mononucleotide.}$$

$$\begin{matrix}HO\diagdown \\ O=P-O\,.\,C_5H_8O_3\,.\,C_4H_3N_2O_2 \\ HO\diagup\end{matrix}\quad \text{uracil mononucleotide.}$$

$$\begin{matrix}HO\diagdown \\ O=P-O\,.\,C_5H_8O_3\,.\,C_4H_4N_3O \\ HO\diagup\end{matrix}\quad \text{cytosine mononucleotide.}$$

$$\begin{matrix}HO\diagdown \\ O=P-O\,.\,C_5H_8O_3\,.\,C_5H_4N_5O \\ HO\diagup\end{matrix}\quad \text{guanine mononucleotide.}$$

The gross structure of nucleic acid would therefore be determined if we could discover the points at which the four nucleotide groups are joined to one another. Only two modes of linkage have sufficient probability to require discussion. One is through the phosphoric acid groups, and the other is through the carbohydrate groups. In the one case, nucleic acid would be a substituted polyphosphoric acid, and in the other case, a substituted

[1] Levene, P. A., and Jacobs, W. A., *Ber. chem. Ges.*, 1909, xlii, 2474.
[2] Levene and Jacobs, *Ber. chem. Ges.*, 1909, xlii, 2703.
[3] Levene and Jacobs, *Ber. chem. Ges.*, 1910, xliii, 3150.
[4] Levene and Jacobs, *Ber. chem. Ges.*, 1911, xliv, 1027.
[5] Levene, P. A., and La Forge, F. B., *Ber. chem. Ges.*, 1912, xlv, 608.
[6] Jones, W., and Richards, A. E., *J. Biol. Chem.*, 1914, xvii, 71.
[7] Jones and Richards, *J. Biol. Chem.*, 1915, xx, 25.

polysaccharide. Prevailing opinion favors the former view, and nucleic acid has been assigned the formula:[3]

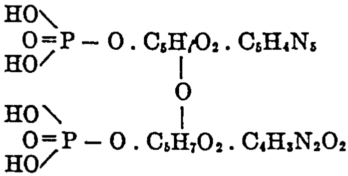
$$
\begin{array}{l}
\mathrm{HO}\diagdown \\
\mathrm{O=P-O\,.\,C_5H_8O_3\,.\,C_5H_4N_5} \\
\qquad | \\
\qquad \mathrm{O} \\
\qquad | \\
\mathrm{O=P-O\,.\,C_5H_8O_3\,.\,C_4H_3N_2O_2} \\
\qquad | \\
\qquad \mathrm{O} \\
\qquad | \\
\mathrm{O=P-O\,.\,C_5H_8O_3\,.\,C_4H_4N_3O} \\
\qquad | \\
\qquad \mathrm{O} \\
\qquad | \\
\mathrm{O=P-O\,.\,C_5H_8O_3\,.\,C_5H_4N_5O} \\
\mathrm{HO}\diagup
\end{array}
$$

The following considerations, however, prove that this is not correct solution. We have prepared from yeast nucleic acid a substance which by acid hydrolysis yields adenine and uracil but neither guanine nor cytosine. By ammoniacal hydrolysis it yields adenosine and uridine but neither guanosine nor cytidine. The partition of its phosphorus shows that it contains an equal number of purine and pyrimidine groups (one of each).[8] The substance is evidently adenine-uracil dinucleotide. Its physical properties are strikingly different from those of yeast nucleic acid and the yield is such as to suggest that its formation from yeast nucleic acid is quantitative.

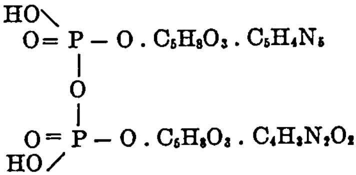
When a hot aqueous solution of the dinucleotide is treated with a solution of brucine in hot alcohol, a tetrabrucine salt is formed which is deposited in beautifully crystalline form as the solution cools. Neither the melting point nor the elementary composition of the brucine salt is changed by repeated crystallization of the substance from hot water. By acid hydrolysis it yields adenine but not guanine. Half of its phosphoric acid is easily split, half is firmly bound. Analyses for carbon, hydrogen, nitrogen, phosphorus, and brucine give sharply the values required for the formula $C_{19}H_{25}N_7P_2O_{15}.(C_{23}H_{26}N_2O_4)_4$.

[8] Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii.

Two structural formulas for a dinucleotide are given below, which differ from one another in only one respect. In I the two mononucleotide groups are joined to one another through their carbohydrate groups, and the formula represents a substituted disaccharide. In II the two mononucleotide groups are joined to one another through their phosphoric acid groups and the formula represents a substituted diphosphoric acid.



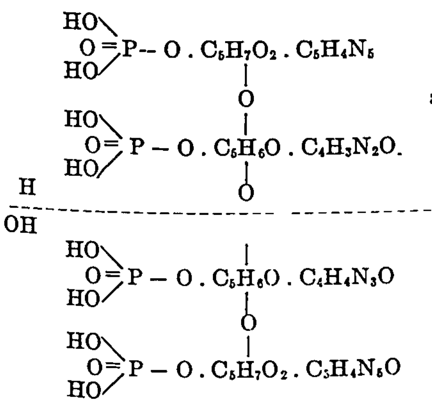
I



II

Formula I can properly be assigned to a dinucleotide that forms a *tetra*brucine salt, and is the most probable formula for the dinucleotide under discussion. Formula II cannot be assigned to a dinucleotide that forms a *tetra*brucine salt and therefore cannot represent the dinucleotide under discussion.

The mode of nucleotide linkage in the dinucleotide must of course exist also in yeast nucleic acid so far as two of its nucleotide groups are concerned, but it does not necessarily follow that this same mode of nucleotide linkage maintains throughout the entire nucleic acid molecule. However, in the following article we shall give evidence to show that such is the case, and that the relation between yeast nucleic acid and adenine-uracil dinucleotide is that which is expressed in the formula·



adenine-uracil dinucleotide.

yeast nucleic acid.

EXPERIMENTAL PART.

Hydrolysis of Yeast Nucleic Acid with Ammonia at 115°, and Separation of the Products.

A solution of 100 gm. of commercial yeast nucleic acid in 530 cc. of 2.5 per cent ammonia was heated for $1\frac{1}{2}$ hours in an autoclave at 115°, and the cooled product was treated with 530 cc. of absolute alcohol. The bulky gelatinous precipitate thus formed was filtered on a Buchner funnel and drawn as completely as possible with a filter pump. The autoclave product is in this way separated into two fractions which may for convenience be designated as the guanine fraction and the adenine fraction. One fraction contains principally guanylic acid and forms an ammonium salt that is almost insoluble in 40 per cent alcohol. The other fraction is almost entirely adenine-uracil dinucleotide, whose ammonium salt is easily soluble in 60 per cent alcohol. When, therefore, a solution of the two fractions in ammonia is treated with an equal volume of alcohol, a sharp separation is effected.

The aqueous alcoholic solution of the dinucleotide was acidified with acetic acid, diluted with an equal volume of hot water, and treated with an aqueous solution of lead acetate as long as the reagent formed a precipitate with a portion of the cooled and filtered solution. About 450 cc. of 25 per cent lead acetate are required. When the precipitation was complete the solution was cooled and the heavy granular lead salt was filtered on a Buchner funnel and washed with a little cold water.

The lead salt was made into a thin paste with boiling water and decomposed with hydrogen sulfide. After the filtrate from lead sulfide had been carefully freed from every trace of hydrogen sulfide by boiling, it was evaporated at 50° under diminished pressure to about 50 cc., and treated with a large amount of absolute alcohol. The precipitated dinucleotide was hardened with absolute alcohol and dried with sulfuric acid in a vacuum desiccator.

From 300 gm. of yeast nucleic acid 174 gm. of the crude dinucleotide were obtained.

Adenine-Uracil Dinucleotide.

Specimens of the dinucleotide prepared in the manner described always contain a small amount of guanylic acid and by acid hydrol-

ysis produce a trace of guanine. The guanylic acid may be completely removed in the following way.

A solution of 100 gm. of the crude dinucleotide in 200 cc. of water is made alkaline with ammonia and treated with 300 cc. of absolute alcohol. The filtered solution is diluted with an equal volume of hot water, acidified with acetic acid, and precipitated with lead acetate as described, but the lead compound is washed with water, until every trace of soluble ammonium salt is removed. This can be done easily and without using the immense volume of wash water that would otherwise be necessary, by grinding up the lead precipitate to a thin paste with boiling water and drawing off the cooled liquid as completely as possible with a filter pump. After the washing in this way has been repeated two or three times, the wash water fails to give off ammonia with sodium carbonate in the cold.

The lead salt is decomposed with hydrogen sulfide, the filtrate from lead sulfide, after boiling until free from the gas, is evaporated, at 50° under diminished pressure, and the pale yellow syrup is treated with a large excess of alcohol. The precipitated dinucleotide is hardened and dried as described.

During the evaporation of the final aqueous solution a persistent bumping (due probably to the absence of ammonium salts) causes a serious loss of material so that the yield is lower than might have been expected. From 100 gm. of the crude dinucleotide 55 gm. of the purified product were obtained, or about 36 per cent of the commercial yeast nucleic acid used. The theoretical yield from pure dry nucleic acid is about 50 per cent. The dinucleotide is an amorphous white powder, soluble in water in all proportions, but insoluble in absolute alcohol. It responds to the color reactions for pentose with orcine and phloroglucine, and by acid hydrolysis yields an abundance of adenine but no trace of guanine.

A solution of 100 mg. of the substance in 2 cc. of 5 per cent sulfuric acid was heated for an hour at 100° and the hot solution was made alkaline with ammonia. No guanine was precipitated even after the solution had stood for a week, but upon the addition of ammoniacal silver nitrate, the fluid became solid with characteristic transparent gelatinous silver adenine. Tests for guanine and adenine made in this way never give misleading results.

The dinucleotide is levorotatory to polarized light. For determining the specific rotation, a preparation was used that had been dried in a vacuum over sulfuric acid and still contained 5 per cent of moisture. A solution of 3.3 gm. in 50 cc. of water gave a reading of $-0.85°$ when polarized in a 2 dm. tube. $[\alpha]_D = -6.8°$.

Preparation of Adenine from the Dinucleotide.—A solution of 5 gm. of dinucleotide in 100 cc. of 5 per cent sulfuric acid was heated for 1 hour at 100° when the product was made alkaline with ammonia and allowed to stand 24 hours. As no precipitation of guanine occurred, the solution was treated with silver nitrate in ammonia and the precipitated gelatinous adenine silver compound was suspended in water and decomposed with hydrogen sulfide. After filtering off the silver sulfide, the solution was evaporated to dryness on the water bath and the residue was crystallized first out of 5 per cent sulfuric acid, then out of hot water with the use of animal charcoal, and finally out of 1 per cent sulfuric acid. There were finally obtained 982 mg. of pure adenine sulfate. The united mother liquors obtained in the crystallization of adenine sulfate were made alkaline with ammonia and boiled until the excess of ammonia had been driven off. The solution was then decolorized with animal charcoal and treated with picric acid. There were finally obtained 189 mg. of adenine picrate. The total adenine obtained from 5 gm. of dinucleotide (5 per cent moisture) was 730 mg. instead of 950 mg.

Preparation of Adenosine from the Dinucleotide.[2]—The dinucleotide was dissolved in five parts of 2.5 per cent ammonia and heated in the autoclave for 2 hours at 135°. As no guanosine was deposited by cooling in ice water, the clear solution was treated with hot concentrated picric acid as long as cold picric acid still gave a precipitate, and the deposited adenosine picrate was recrystallized from hot water. From 15 gm. of dinucleotide we obtained 5.27 gm. of the picrate in glistening plates.

The picrate was dissolved in hot water and after the solution had cooled it was made and kept acid to Congo red with sulfuric acid as it was shaken out with ether for the removal of picric acid. The sulfuric acid was then neutralized with lead carbonate,[9] and

[9] This was to avoid the continual deposition of a barium compound during the subsequent evaporation, as is the case when barium carbonate is used.

the filtered solution was treated with hydrogen sulfide for the precipitation of a trace of lead. After evaporation of the filtrate from lead sulfide at 50° under diminished pressure and further concentration over sulfuric acid, the fluid was allowed to remain over night in the ice chest. A paste of crystalline adenosine was formed, from which pure adenosine was prepared in needle clusters, by recrystallization from hot water with the use of animal charcoal.

The substance was ash-free, contained no trace of free adenine, melted to a black liquid sharply at 219° (corrected), and contained one and one-half molecules of water of crystallization of which one molecule is driven off by heating in the air at 115°.[10]

0.3408 gm. air-dried substance lost 0.0210 gm. at 115°.

Calculated for $C_{10}H_{13}N_5O_4.\frac{1}{2}H_2O.H_2O$:	Found:
6.12	6.16

I. 0.1945 gm. dried at 115° required 13.35 cc. H_2SO_4 (1 cc. = 0.00370 gm. N).

II. 0.1233 gm. dried at 115° required 8.47 cc. H_2SO_4.

	Calculated for $C_{10}H_{13}N_5O_4.\frac{1}{2}H_2O$:	Found: I.	Found: II.
N	25.36	25.39	25.42

The picrate prepared from pure adenosine formed thin transparent plates with sharp edges, which decomposed and melted at 183.5° (corrected).

Preparation of Uracil from the Dinucleotide.—31 gm. of dinucleotide were heated in an autoclave at 140° for $3\frac{1}{2}$ hours with 190 cc. of 25 per cent sulfuric acid. The product was diluted to 1 liter and precipitated with an excess of hot concentrated barium hydroxide. After filtration the excess of barium was removed by passage of carbon dioxide and the solution was evaporated under diminished pressure to about 250 cc. The concentrated fluid was acidified with nitric acid, the purine bases (adenine and hypoxanthine) were removed from the acid solution by the addition of silver nitrate, and the uracil was precipitated in the usual way with silver nitrate and barium hydroxide. The silver com-

[10] Levene and La Forge drove off one and one-half molecules of water from adenosine by heating at 110° in a vacuum over phosphorus pentoxide.

pound of uracil was suspended in hot water and decomposed with hydrogen sulfide. By evaporating the filtrate from silver sulfide to crystallization, there were obtained 5.18 gm. of crude uracil. This was recrystallized twice from 5 per cent sulfuric acid and finally from water with the use of animal charcoal. 2.618 gm. of pure uracil were obtained in macrocrystalline needles.

I. 0.2333 gm. required 15.78 cc. H_2SO_4 (1 cc. = 0.00370 gm. N).
II. 0.3262 " " 21.91 " "

	Calculated for $C_4H_4N_2O_2$:	Found: I.	II.
N	25.00	25.03	24.85

All fluids obtained in the purification of uracil were joined to the first mother liquor and examined for cytosine, but with negative results.

Preparation of Uridine from the Dinucleotide.[5]—A solution of dinucleotide in 2.5 per cent ammonia was heated in the autoclave for 2 hours at 133°. The product did not deposit guanosine when cooled in ice water for 2 hours, nor even upon standing over night in the ice chest. After warming to the room temperature the clear solution was treated with hot concentrated aqueous picric acid, as long as a drop of the fluid removed for the purpose was still found to give a precipitate with cold saturated picric acid. After completely precipitating the adenosine in this way, the solution was cooled and filtered. The excess of picric acid was removed with sulfuric acid and ether, the sulfuric acid was precipitated with hot concentrated barium hydroxide, and the solution was evaporated almost to dryness at 50° under diminished pressure. Alcohol was added, hydrochloric acid was passed in to the point of saturation, and the product was further treated as Levene and La Forge direct.[5]

Owing to the complication of the process we made no attempt to obtain a quantitative yield.. From 100 gm. of air-dried dinucleotide there were obtained 7.3 gm. of snow-white crystalline uridine. (The yield from yeast nucleic acid is 5 per cent.)

I. 0.3883 gm. required 11.89 cc. H_2SO_4 (1 cc. = 0.00370 gm. N)
II. 0.3212 " " 10.02 " "

	Calculated for $C_9H_{12}N_2O_6$:	Found: I.	II.
N	11.47	11.33	11.54

The substance melted at 159° (corrected). Specimens of pure uridine from the dinucleotide and from yeast nucleic acid were heated in two capillary tubes on the same thermometer. The two specimens melted simultaneously at 159° (corrected).

Cytidine was not present in the first mother liquor obtained in the preparation of uridine. (When dealing with yeast nucleic acid, cytidine nitrate can always be prepared from this mother liquor.)

The Uracil Group in the Dinucleotide.—It has been suggested that the existence of a uracil group in nucleic acid has never been proven, and this is really the case if rigid proof be demanded. It is true that both uracil and uridine can be obtained from nucleic acid, but the corresponding amino compounds cytosine and cytidine are always found at the same time. The two oxy-compounds can easily be formed from the amino-compounds and might actually be produced in this way during the acid hydrolysis of nucleic acid, for neither of the oxy-compounds has been obtained from nucleic acid without employing hot mineral acid either in the hydrolysis itself or in the isolation of the product.

The failure to find any trace of cytosine among the hydrolytic products of the dinucleotide is evidence of considerable value but the question is finally decided by the chemical composition of the crystalline brucine salt of the dinucleotide.

Nitrogen required for the brucine salt of adenine-*cytosine* dinucleotide		10.05
Nitrogen required for the brucine salt of adenine-*uracil* dinucleotide		9.42
Nitrogen found.	I	9.35
	II	9.41
	III	9.35

A uracil group therefore exists in the dinucleotide and must also exist in the nucleic acid.

Partition of Phosphorus in the Dinucleotide.[8]—This subject is dealt with exhaustively in the contribution that follows,[11] where it is shown that half of the phosphoric acid comes off by mild hydrolysis with dilute mineral acid while half remains firmly bound. As far as this has evidential value it shows that the dinucleotide

[11] Jones, W., and Read, B. E., *J. Biol. Chem.*, 1917, xxix, 123.

is a mixed dinucleotide composed of groups of two mononucleotides, one a purine nucleotide and the other a pyrimidine nucleotide.

The Brucine Salt of the Dinucleotide.

A solution of 10 gm. of dinucleotide in 30 cc. of hot water was treated with a solution of 24.2 gm. of brucine in 48.4 cc. of hot alcohol. Crystals of the brucine salt appeared almost immediately. When the material had cooled to the room temperature, the crystals were filtered off, washed first with cold water, then with hot alcohol, and allowed to dry in the air. The salt weighed 21.12 gm. After recrystallization from fifty parts of hot water 10.4 gm. were obtained.

By repeated crystallization of the compound from hot water there is a considerable loss of material, due to its solubility in cold water. This can be recovered by careful evaporation of the mother liquors at a low temperature, but there is little need for recrystallization since the crystalline salt first obtained has the same melting point and elementary composition as the products of its recrystallization. The recrystallized salt was used in the analytical work reported in the next section.

When heated in a capillary tube the brucine salt contracts and recedes from the sides of the tube at 172–173°, but is not otherwise changed until the temperature reaches 174°. Between 174° and 175° the substance turns brown and melts. This same conduct is exhibited by all specimens of the brucine salt even after recrystallization from hot water five times. The hydrous salt contains fourteen molecules of water of crystallization and has the composition represented by the formula $C_{19}H_{25}N_7P_2O_{15}.4(C_{23}H_{26}N_2O_4).14H_2O$. The substance is very stable in the air at the room temperature, but slowly loses weight in a vacuum over sulfuric acid. Heated in the air at 110° it loses all of its water of crystallization and the weight of the anhydrous salt is not changed either by heating several hours longer at 125° or by heating at 110° in a vacuum over phosphorus pentoxide. The anhydrous salt takes up water and increases in weight when exposed to the air.

The material used for the following analyses was recrystallized from hot water once or oftener and except for the determinations of water of

crystallization was dried at 115°. The required values in the table below are for the formula $C_{19}H_{25}N_7P_2O_{15}.4(C_{23}H_{26}N_2O_4)$ except those for water of crystallization which are calculated from the formula $C_{19}H_{25}N_7P_2O_{15}.4(C_{23}H_{26}N_2O_4).14H_2O$. In determining the phosphorus, the substance was oxidized with potassium sulfate and concentrated sulfuric acid to which a few drops of 20 per cent copper sulfate were added.

For determining the brucine, the salt was suspended in fifty parts of hot water, and ammonia was added, until the substance passed into solution. Upon cooling, crystalline brucine was deposited, $C_{23}H_{26}N_2O_4.4H_2O$. This was filtered off and the filtrate was extracted with chloroform which was evaporated in a weighed platinum dish. The crystalline brucine was added and after drying at 110° the dish was again weighed. About 97 per cent of the brucine is precipitated by ammonia in crystalline form and is easily soluble in chloroform; about 3 per cent remains for the chloroform extraction.

I. 0.6219 gm. air-dried substance lost 0.0636 gm. at 115°.
II. 0.4947 " " " " 0.0502 " " 115°.
III. 0.2401 " dried at 115° gave 0.1269 gm. H_2O and 0.5265 gm. CO_2.
IV. 0.2561 " gave 0.1299 gm. H_2O and 0.5588 gm. CO_2.
V. 0.3596 " " 30.0 cc. N (25° and 768 mm.).
VI. 0.3920 " " 33.1 " N (26.5° " 760 ").
VII. 0.3418 " " 28.4 " N (25° " 766.5 ").
VIII. 1.3874 " " 0.1389 gm. $Mg_2P_2O_7$.
IX. 1.4243 " " 0.1419 " "
X. 1.2254 " " 0.2704 " $MgNH_4PO_4.6H_2O$.
XI. 0.4445 " " 0.3114 " brucine.
XII. 0.4756 " " 0.3344 " "

	H_2O	C	H	N	P	Brucine.
Calculated.	10.16	59.76	5.79	9.42	2.78	70.7
Found.						
I.........	10.23					
II.........	10.14					
III.........		59.80	5.87			
IV.........		59.51	5.64			
V.........				9.41		
VI.........				9.35		
VII.........				9.35		
VIII.........					2.79	
IX.........					2.77	
X.........					2.79	
XI.........						70.1
XII.........						70.3

Partition of Phosphorus in the Brucine Salt.[8]—Accurate determination of the partial phosphoric acid in the brucine salt is difficult because the brucine not only disturbs the hydrolysis but also interferes with the estimation of the products. The results therefore differ somewhat from the theoretical.

After first making a determination of total phosphoric acid, a specimen of the brucine salt was heated at 100° for 1 hour with twenty parts of 10 per cent sulfuric acid. The fluid, made alkaline with ammonia, was cooled, and filtered from precipitated brucine. The filtrate was treated with magnesia mixture and the ammonium magnesium phosphate weighed. From the amount of this substance obtained, the pyrimidine correction for 1 hour was subtracted.

1.2254 gm. gave 0.2704 gm. $MgNH_4PO_4.6H_2O$ (total).
0.9049 " " 0.1131 " " (partial).

The results are expressed in percentages of magnesium ammonium phosphate.

Total found	22.06
" required	22.00
Partial found	11.49
" required	11.00

THE MODE OF NUCLEOTIDE LINKAGE IN YEAST NUCLEIC ACID.

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When a purine nucleotide is heated with dilute sulfuric acid its phosphoric acid is set free rapidly and completely. On the contrary, a pyrimidine nucleotide loses its phosphoric acid under the same conditions very slowly, so that the existence of purine and pyrimidine groups in mixed nucleotides can be indirectly ascertained from the rapidity with which their phosphoric acid is set free. Thus, yeast nucleic acid is a tetranucleotide composed of groups of two purine nucleotides and two pyrimidine nucleotides. As we should expect, half of its phosphoric acid is easily set free and half is firmly bound.

In the following are given the results obtained by the application of this method to adenine-uracil dinucleotide.

EXPERIMENTAL.

Eight portions of dinucleotide were heated with 5 per cent sulfuric acid (20 cc. per gm. of substance) for various periods of time from ½ hour to 7 hours, and the liberated phosphoric acid was determined as magnesium ammonium phosphate. In addition, the total phosphoric acid of the dinucleotide was determined after destroying the organic matter. The details of this procedure are described in former articles.[1] The results are given in Table I and for comparison the results formerly obtained with yeast nucleic acid are given in Table II.

These results are represented diagrammatically in Figs. 1 and 2. Hours are laid off as abscissæ and amounts of magnesium ammonium phosphate multiplied by 5 are laid off as ordinates. In Fig. 1 the upper curve is the locus of points that represent the

[1] Jones, W., *J. Biol. Chem.*, 1916, xxiv, p. iii; 1916, xxv, 87. Germann, H. C., *ibid.*, 1916, xxv. 189.

TABLE I.

Hydrolysis of Adenine-Uracil Dinucleotide.

Substance used.	Corrected for 3 per cent of moisture.	Time of heating.	Magnesium ammonium phosphate obtained.	Amount per gm. of dinucleotide.	Pyrimidine correction.	Amount from the purine nucleotide.
gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*mg.*	*gm.*
0.6536	0.6340	½	0.1339	0.211	5	0.206
0.6450	0.6256	1	0.1790	0.286	10	0.276
0.8110	0.7867	2	0.2724	0.346	20	0.326
0.7738	0.7506	3	0.2704	0.360	30	0.330
0.8148	0.7904	4	0.2976	0.377	40	0.337
0.8378	0.8127	5	0.3102	0.382	50	0.332
0.8155	0.7910	6	0.3129	0.396	60	0.336
0.9627	0.9338	7	0.3750	0.402	70	0.332
0.6178	0.5993	Total.	0.4012	0.669	½ total =	0.335
0.5932	0.5754	"	0.3883	0.675		0.338

TABLE II.

Hydrolysis of Yeast Nucleic Acid.

Substance used.	Corrected for 8.5 per cent of moisture.	Time of heating.	Magnesium ammonium phosphate obtained.	Amount per gm. of nucleic acid.	Pyrimidine correction.	Amount from purine nucleotides
gm.	*gm.*	*hrs.*	*gm.*	*gm.*	*mg.*	*gm.*
0.7347	0.6723	½	0.1284	0.191	5	0.186
0.8644	0.7909	1	0.2315	0.292	10	0.282
0.9837	0.8991	2	0.3125	0.348	20	0.328
0.9251	0.8465	3	0.3038	0.359	30	0.329
1.0305	0.9429	4	0.3488	0.371	40	0.331
0.8333	0.7625	5	0.2876	0.377	50	0.327
0.9927	0.9083	6	0.3565	0.392	60	0.332
0.9667	0.8845	7½	0.3625	0.410	75	0.335
0.8179	0.7484	Total.	0.5112	0.644	½ total =	0.322

amount of magnesium ammonium phosphate actually obtained from the dinucleotide and it shows the rate at which phosphoric acid is liberated from both mononucleotide groups. The several points of the lower curve are obtained by calculation, *i.e.*, by subtraction of the pyrimidine correction, so that the curve shows the rate at which phosphoric acid is liberated from the purine nucleotide group. In Fig. 2 yeast nucleic acid is similarly represented.

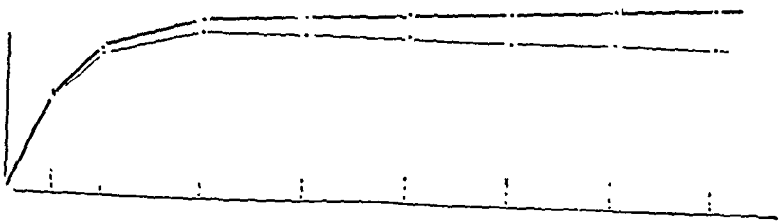


FIG. 1.

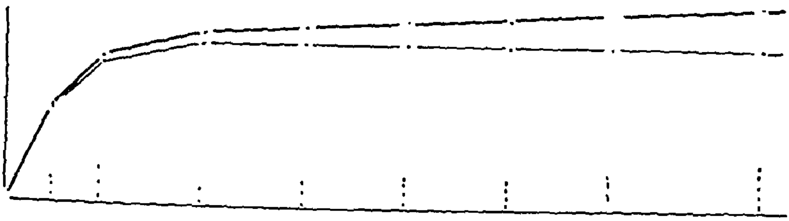
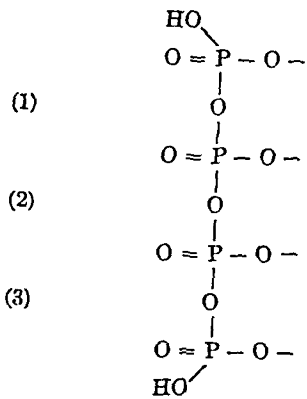


FIG. 2.

CONCLUSION.

The two figures are as nearly superimposable upon one another as could reasonably be expected. Therefore, so far as concerns its phosphoric acid linkages (but no farther), nucleic acid is composed of the groups of two dinucleotides identical with one another and identical with the nucleic acid itself. Hence the phosphoric acid linkage (2) in the diagram below does not exist.



Again, the two dinucleotides must have identical phosphoric acid linkages. If linkage (1) exists, linkage (3) must also; if linkage (1) does not exist, linkage (3) cannot. But it was shown in the preceding article that there is no direct phosphoric acid linkage in adenine-uracil dinucleotide and therefore none at the corresponding point in yeast nucleic acid. Hence both linkages (1) and (3) are excluded and there is no direct linkage of phosphoric acid groups in the entire nucleic acid molecule.

That the nucleotide groups of yeast nucleic acid (and adenine-uracil dinucleotide as well) are not united to one another through their phosphoric acid groups is proven. That this union is through the carbohydrate groups, we conclude from the general principles of physiological chemistry. The combination of purine groups with pyrimidine groups or of either with a carbohydrate group whose aldehyde affinity is already satisfied is very rare in physiological chemistry. But the polysaccharide structure is sufficiently common.

It is of course possible to draw other conclusions that can be adjusted to the facts, but they all involve the assumption of curious coincidences and compensations which would cause phosphoric acid to be liberated with equal ease from different kinds of linkage, or that the liberation of phosphoric acid from one kind of linkage is excessively slower than from another. After careful consideration of such matters, we believe we have drawn the correct conclusion.

THE INFLUENCE OF INTRAVENOUS INJECTION OF WITTE'S PEPTONE UPON THE SUGAR CONTENT OF THE BLOOD AND EPINEPHRINE HYPERGLYCEMIA AND GLYCOSURIA.

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(Received for publication, December 11, 1916.)

Henderson and Underhill reported that the intravenous injection of peptone into dogs causes hyperglycemia and glycosuria which they considered due to an accompanying acapnia. More recently McGuigan and Ross have stated that peptone administered to dogs intravenously induces a notable hypoglycemia and they ascribe the results obtained by Henderson and Underhill to anesthesia.

On the other hand, Glaessner and Pick claim that both pancreatic juice and peptone can inhibit epinephrine glycosuria. If Glaessner and Pick's report is compared with that of McGuigan and Ross, it is possible that the inhibitory influence of peptone upon epinephrine glycosuria may be due to the hypoglycemia caused by peptone. But it is also possible that peptone decreases the permeability of the kidney for sugar and thus inhibits epinephrine glycosuria, notwithstanding that the blood sugar may or may not be changed simultaneously. Though pancreatic extract has been shown by many investigators to have an inhibitory influence upon epinephrine glycosuria, Leschke demonstrated that an intravenous injection of pancreatic extract alone induces glycosuria. At the suggestion of Professor Frank P. Underhill, I have investigated the effect of peptone injection upon the sugar content of the blood and also upon epinephrine hyperglycemia and glycosuria.

Methods.

Full-grown rabbits were used. They were fed on oats and corn, greens being added to the diet from time to time. Water was

given freely. The urine was obtained by pressure on the bladder through the abdominal wall.

Witte's peptone was always employed in 10 per cent solution in 0.9 per cent sodium chloride solution. Unless otherwise noted, the peptone solution was always sterilized by boiling. Peptone (0.3 to 0.75 gm. per kilo of body weight) was injected into an ear vein without anesthesia. Blood samples for sugar determination were drawn from the other ear. For the control experiments an equal volume of 0.9 per cent sodium chloride solution was used. McGuigan and Ross assert that gelatin and other proteins can cause hypoglycemia as well as peptone. In accordance with this statement some experiments have been made to test the influence of gelatin and egg albumin upon blood sugar content. Commercial gelatin and a preparation of egg albumin were made up to 10 per cent solution with 0.9 per cent sodium chloride solution. The gelatin solution was sterilized by boiling, the egg albumin solution being used without sterilization.

When peptone and epinephrine were injected successively, peptone solution was first injected into an ear vein and immediately after that epinephrine (adrenalin chloride 1:1000 Parke, Davis and Company) was administered subcutaneously.

The influence of intravenous injection of gelatin, egg albumin, and also soluble starch upon epinephrine hyperglycemia and glycosuria was studied in some experiments. The methods were the same as described with peptone and epinephrine. Soluble starch (Kahlbaum) was injected in 10 per cent solution, being dissolved in 0.9 per cent sodium chloride solution and sterilized by boiling.

In some cases body temperature, respiration, and pulse rate were determined. The body temperature was measured by inserting a thermometer into the rectum after collection of the blood samples. Respiration and pulse rate were measured directly before the taking of a blood sample.

The blood sugar was determined by the Lewis-Benedict method, as described by Miss McDanell in this laboratory. The sugar in the urine was estimated by a Schmidt and Haensch triple shadow saccharimeter after removal of the coloring matters and levorotatory substances by a saturated solution of mercuric acetate. Peptone, gelatin, and egg albumin added to the urine were shown not to interfere with this method. For the qualitative tests of sugar in the urine Benedict's reagent was employed.

The Influence of Intravenous Injection of Witte's Peptone upon the Sugar Content of the Blood.

When Henderson and Underhill injected Witte's peptone (0.3 to 0.63 gm. per kilo of body weight) intravenously into dogs, the sugar content of the blood increased as high as 0.26 to 0.27 per cent and the urine samples contained large amounts of sugar. McGuigan and Ross also injected Witte's peptone intravenously into dogs, using doses of about 0.12 to 0.66 gm. per kilo of body weight. In their experiments the sugar content of the blood reached a minimum about 2 hours after the peptone injection. The usual result was a fall of the sugar to about one-half or one-third of the original, which in general was much lower than that recorded by other investigators. In some cases they observed a transient and to them insignificant hyperglycemia in the interval between the injection of peptone and the hypoglycemic state; they ascribe this period of hyperglycemia to asphyxial conditions. Henderson and Underhill specifically called attention to the fact that the injection of Witte's peptone induces respiratory disturbances which they ascribed as being the cause of the hyperglycemia and glycosuria present. It is evident that McGuigan and Ross have confirmed the contention of Henderson and Underhill who made no statement concerning blood sugar content or glycosuria after peptone injection so arranged as to have no relation to respiratory disturbances. It is a well known fact that peptone is toxic, when administered intravenously (Underhill, Popielski). Underhill (1903) investigated its behavior with material of both animal and vegetable origin. The susceptibility to peptone is not the same in different species of animals. An intravenous administration of peptone usually brings forth severe toxic symptoms in dogs and sometimes kills the animals even in the dose used by Henderson and Underhill or McGuigan and Ross. The rabbit is usually considered to be less susceptible to peptone. Buchner and Geret reported that an intraperitoneal injection of pure peptone, specially prepared by themselves, in doses of 0.2 gm. per kilo of body weight, killed rabbits within 12 hours. In my own experiments the rabbits usually showed no severe symptoms except a slight degree of prostration after an intravenous injection of Witte's peptone in doses of 0.5 to 0.75 gm. per kilo of body weight. In a few cases 0.5 to 1.0 gm. of Witte's peptone per kilo

of body weight killed the rabbits immediately or in a short time. In such cases prostration, weak heart action, and convulsions were the most noticeable symptoms. The results of the experiments are shown in Table I.

TABLE I.

The Influence of Intravenous Injection of Witte's Peptone upon the Sugar Content of the Blood.

Rabbit. No.	Rabbit. Body weight.	Amount of peptone injected per kilo of body weight.	Blood sugar content (percentage). Before injection.	Hrs. after injection. $\frac{1}{4}$	$\frac{1}{2}$	1	2	3	4	6	8	24	48
	kg.	*gm.*											
I	2.50	0.5	0.15	0.22		0.18	0.13	0.12	0.11	0.11		0.13	0.14
II	1.88	0.5	0.13		0.18	0.21	0.20	0.15	0.12	0.12	0.12	0.14	0.12
III	1.98	0 5	0.13	0.17	0.17	0.15	0.15	0.13	0.13	0.12	0.13	0.13	0.14
IV	1.84	0.5	0.14	0.15	0.19	0.23	0.20	0.18	0.14		0.11		
V	1.96	0.5	0.14		0.19	0.17	0.13	0.12	0.13				
VI	2.18	0.5	0.11			0.15	0.18	0.12	0.12				
VII	2.18	0.5	0.10			0.11	0.12	0.13	0.10				
VIII	2.80	0.75	0.11			0.18	0.16	0.12	0.10				
IX	1.90	0.75	0.12			0.16	0.16	0.12	0.10				
Average...........			0.13	0.18	0.18	0.17	0.16	0.13	0.12	0.12	0.12	0.13	0.13

Respiration (R), Pulse Rate (P), and Body Temperature (T).

Rabbit.		Before injection.	Hrs. after injection. $\frac{1}{4}$	$\frac{1}{2}$	1	2	3	4	6	8	24	48
II	R.	88		92	90	88	92	120	100	120	98	80
	P.	192		200	203	230	254	200	254	240	240	252
	T.	39.6		39.2	39.0	39.0	38.9	39.3	39.5	39.6	39.2	38.8
III	R.	88		100	60	68	72	88	88	88	100	68
	P.	140		148	200	192	164	192	168	168	180	160
	T.	38.1		38.3	38.8	38.4	38.6	39.1	39.3	38.5	38.8	38.5
IV	R.	68		76	80	80	68	80		76		
	P.	220		262	240	236	228	240		252		
	T.	38.5		38.4	38.6	38.7	38.9	39.2		39.4		
V	R.	120		80	88	92	72	72				
	P.	156		180	216	216	240	240				
	T.	38.3		38.1	38.4	38.6	39.3	39.0				

From the data in Table I, it is evident that *in the rabbit hypoglycemia is not induced by the intravenous injection of Witte's peptone, but, on the contrary, in most cases a slight hyperglycemia is invoked a few hours after the injection. Glycosuria does not develop.* Peptone injection had no remarkable influences upon the body temperature, respiration, and pulse rate. An excessive respiration, which was noticed by Henderson and Underhill in dogs, could not be demonstrated distinctly by rabbit experiments. The estimation of blood gases was not made in the present experiments. It cannot be stated here, therefore, whether the slight hyperglycemia, which was found in most cases of my experiments, is also due to acapnia or not.

To eliminate a possible error, which might be caused by the peptone itself circulating in the blood, a small amount of peptone was added to blood samples *in vitro,* and the sugar content of the mixture was estimated. The results showed that the method employed for the sugar determination was not interfered with by peptone itself.

Sodium chloride can cause glycosuria (salt glycosuria). But in order to obtain such results, a large amount of saline solution must be used. Underhill and Closson observed glycosuria after injecting about 90 cc. of $\frac{1}{2}$ molecular sodium chloride solution. In their experiments the glycosuria was accompanied by hypoglycemia. Bang could find neither glycosuria nor hyperglycemia after injecting about 100 cc. of 0.9 per cent NaCl solution (10 cc. in 10 minutes). It is a well known fact that emotion sometimes causes glycosuria. Cannon, Shohl, and Wright demonstrated that simply binding fast is enough to cause glycosuria in a cat. But nobody has observed such emotional glycosuria in rabbits. In Table II, two control experiments with 0.9 per cent sodium chloride solution instead of peptone solution are shown. The sugar content of the blood, respiration, pulse rate, and body temperature were not markedly affected.

McGuigan and Ross demonstrated that anesthesia can cause hyperglycemia. Epstein, Reiss, and Branower also reported that operations under anesthesia cause hyperglycemia in human beings; for example, before anesthesia the blood sugar content was 0.1 to 0.13 per cent, and after operation 0.17 to 0.27 per cent. In my experiments anesthetics were omitted. A hyperglycemia was also

TABLE II.

Control Experiments with the Saline Solution.

Rabbit.		Volume of 0.9 per cent NaCl solution injected.	Blood sugar content (percentage).					
No.	Body weight.		Before injection.	Hrs. after injection.				
				1	2	3	4	6
	kg.	*cc.*						
I	2.88	14.4	0.11	0.13	0.13	0.11	0.11	0.11
II*	2.80	21.0	0.13	0.11	0.12	0.12	0.11	0.11

Respiration (R), Pulse Rate (P), and Body Temperature (T).

Rabbit.		Before injection.	Hrs. after injection.				
			1	2	3	4	6
I	R.	80	76	88	100	104	96
	P.	240	252	276	240	264	264
	T.	38.7	39.1	39.2	39.4	39.0	38.5
II*	R.	84	80	88	88	92	104
	P.	216	252	240	240	240	264
	T.	38.8	38.6	38.7	38.6	38.6	39.3

* This was Rabbit VIII in Table I.

demonstrated after a considerable loss of blood (Bang). In my experiments the whole amount of blood taken for sugar estimation was not large enough for consideration of such a factor.

McGuigan and Ross thought that peptone is more active in causing a lowering of the blood sugar when it is dissolved in cold water and not boiled after solution. In order to find out if there is any such difference between unboiled and boiled peptone, unboiled peptone was injected into two rabbits. Here also, instead of lowering blood sugar, the level was more apt to increase (Table III).

After intravenous administration of peptone the sugar content of the blood sometimes increased as high as 0.2 per cent or more (Rabbits I, II, and IV in Table I). But in none of the cases was glycosuria observed. When Epstein and his coworkers found a slight hyperglycemia after operations under anesthesia they could not demonstrate any glycosuria. By testing the renal function with phenolsulfonephthalein in such cases, they found that

TABLE III.

The Influence of Intravenous Injection of Unboiled Peptone upon the Sugar Content of the Blood.

Rabbit.		Amount of peptone injected per kilo of body weight.	Blood sugar content (percentage).				
			Before injection.	Hrs. after injection			
No.	Body weight.			1	2	3	4
	kg.	*gm.*					
I	2.14	0.5	0.10	0.12	0.12	0.17	0.10
II	1.86	0.5	0.12	0.15	0.15	0.14	0.14

the absence of glycosuria is due to a change of the renal permeability. In the case of peptone this explanation for the absence of glycosuria seems to be plausible, as a later description shows.

The Influence of Intravenous Injection of Gelatin and Egg Albumin upon the Sugar Content of the Blood.

McGuigan and Ross asserted that lowering of the blood sugar is the usual result of the intravenous injection of proteins, and this was proved by blood transfusion and the injection of gelatin. The results of my experiments in this direction are shown in Table IV.

TABLE IV.

The Influence of Intravenous Injection of Gelatin and Egg Albumin upon the Sugar Content of the Blood.

Rabbit.		Amount of substance injected per kilo of body weight.	Blood sugar content (percentage).				
			Before injection.	Hrs. after injection.			
No.	Body weight.			1	2	3	4
Gelatin.							
	kg.	*gm.*					
I	1.84	0.5	0.11	0.11	0.10	0.10	0.10
II	2.20	0.5	0.12	0.12	0.13	0.13	0.12
III	2.20	0.75	0.11	0.11	0.10	0.12	0.11
Egg albumin.							
IV	2.20	0.5	0.12	0.13	0.11	0.13	0.11
V	1.94	0.5	0.12	0.15	0.15	0.15	0.13
VI	1.94	0.75	0.11	0.10	0.09	0.09	0.09
VII	2.00	0.75	0.12	0.11	0.12	0.14	0.12

From these results it is apparent that *no noticeable influence of gelatin and egg albumin upon blood sugar content could be observed.* Neither glycosuria nor any severe disturbance of the general condition of the animals was observed.

The Influence of Intravenous Injection of Witte's Peptone upon Epinephrine Hyperglycemia and Glycosuria.

There are many substances which are said to inhibit epinephrine glycosuria. Zuelzer, and Biedl and Offer reported that they succeeded in inhibiting epinephrine glycosuria by injecting pancreas preparations. Biedl and Offer also obtained the same result by injecting the thoracic duct lymph. Glaessner and Pick inhibited epinephrine glycosuria by injecting pancreatic juice or Witte's peptone. Investigating the relations of the ductless glands, Eppinger, Falta, and Rudinger explained Zuelzer's experiments by saying that the excess sugar mobilized by epinephrine was oxidized by the pancreas extract injected simultaneously and therefore was not eliminated in the urine.

It is interesting to observe that Leschke induced glycosuria by injecting pancreatic extract, which is said to inhibit epinephrine glycosuria. Tomaszewski and Wilenko reported that sodium chloride solution, which acts as a lymphagogue, can inhibit epinephrine glycosuria. As to the influence of calcium chloride upon epinephrine glycosuria, the results of investigators are not concordant. Although Schrank demonstrated an inhibitory influence of this salt, Underhill (1916) was able to cause a noticeable increase of epinephrine glycosuria. In Underhill's experiments, after subcutaneous injection of calcium salt, epinephrine hyperglycemia was maintained, but its curve was distinctly modified. After confirming the results of Zuelzer, Biedl and Offer, and others, von Fürth and Schwarz succeeded in inhibiting epinephrine glycosuria by intraperitoneal injection of turpentine or aleuronat. They estimated the sugar content of the blood, nitrogen, sodium chloride, and sugar content of the urine, and reached the conclusion that the inhibitory influence of pancreas preparations, turpentine, or aleuronat upon epinephrine glycosuria is not specific, but is due to the damage of the renal function. They added that the damage of the kidneys cannot be judged by the amount of

urine alone, as in some cases the amount was not at all affected, while the glycosuria was markedly decreased.

So far as I am aware, the only investigation of the influence of peptone upon epinephrine glycosuria is that reported by Glaessner and Pick. According to their description, when a dog weighing 6.8 kg. received 5 cc. of epinephrine solution subcutaneously, 5 gm. of sugar were found in 425 cc. of the urine, but 4 days later when it received 2 gm. of Witte's peptone intravenously, and 5 cc. of epinephrine solution subcutaneously, no sugar was found in 400 cc. of the urine. The blood sugar was not estimated.

My experiments were made with rabbits and the results are detailed in Table. V. As some rabbits could not tolerate 1 mg. of epinephrine and 0.5 gm. of peptone per kilo of body weight, the amount of peptone was reduced to 0.3 to 0.35 gm. per kilo of body weight.

It is to be noted from Table V that *the grade of hyperglycemia produced by epinephrine was not markedly changed by an intrave-*

TABLE V.

The Influence of Intravenous Injection of Witte's Peptone upon Epinephrine Hyperglycemia and Glycosuria.

Rabbit.		Amount of substance injected per kilo of body weight.		Blood sugar content (percentage).						Urine (in 7½ hrs.).		
				Before injection.	Hrs. after injection.							
No.	Body weight.	Epinephrine.	Peptone.		1½	3	4½	6	7½	Volume.	Sugar.	
	kg.	*mg.*	*gm.*							*cc.*	*per cent*	*gm.*
IA*	2.06	1.0		0.10	0.31	0.32	0.31	0.28	0.15	128	3.31	4.24
IB	2.08	1.0	0.3	0.11	0.27	0.30	0.29	0.27	0.19	108	0.48	0.51
IC	2.04	1.0				0.32	0.34			100	3.20	3.20
II	1.84	1.0		0.13	0.35	0.32	0.30	0.25	0.20	140	2.50	3.53
III	1.64	1.0	0.5	0.12	0.32	0.30	0.13	0.10	0.09	144	0.19	0.27
IV	1.92	1.0	0.5	0.11	0.26	0.24	0.32	0.36	0.32	49	0.61	0.30
VA**	2.16	1.0	0.35	0.11	0.28	0.30	0.33	0.31	0.26	60	0.21	0.13
VB	2.10	1.0				0.36	0.33			53	3.94	2.09

* Three experiments, IA, IB, and IC, were carried through with Rabbit I at intervals of 1 week.

** Two experiments, VA and VB, were carried through with Rabbit V with an interval of 1 week.

nous injection of peptone. But epinephrine glycosuria was distinctly decreased by peptone. Averaging the amounts of sugar in the urine, *3.3 gm.* of sugar were eliminated after the injection of epinephrine alone, while the amount of sugar was only *0.3 gm.* when peptone was injected simultaneously. It is a well known fact that the same dose of epinephrine may not produce the same degree of glycosuria in two different animals or in the same animal at different periods. But this seems to be insufficient to explain the data described above. The explanation of the decrease of epinephrine glycosuria, suggested by von Fürth and Schwarz, when pancreas preparations, turpentine, or aleuronat were injected simultaneously, *i.e.*, the damage of the renal function, may be applied here. Their statement that the renal permeability for sugar cannot be judged by the amount of urine only is also here confirmed. The absence of glycosuria, where a significant hyperglycemia was caused by administration of peptone alone (Table I), may also be explained in the same manner.

The Influence of Intravenous Injection of Gelatin, Egg Albumin, and Soluble Starch upon Epinephrine Hyperglycemia and Glycosuria.

When gelatin or egg albumin was injected intravenously just before epinephrine (1 mg. per kilo of body weight) injection, the amount of both gelatin and egg albumin had to be reduced so as to be less than 0.2 gm. per kilo of body weight. All rabbits in which gelatin or egg albumin was injected with a dose over 0.2 gm. per kilo of body weight died immediately or within a few hours. The heart action was markedly affected. It is conceivable that the colloidal nature of the protein injected may be responsible for the death of the animals after epinephrine introduction. To determine this point experiments have been carried through with a colloidal solution of a different nature, soluble starch. When soluble starch was employed in place of gelatin or egg albumin the general condition of the animal was not changed. The results are shown in Table VI.

It is to be noted from Table VI that *the grade of hyperglycemia called forth by epinephrine was not markedly changed by the intravenous injection of gelatin or egg albumin, but epinephrine gly-*

TABLE VI.

The Influence of Intravenous Injection of Gelatin, Egg Albumin, and Soluble Starch upon Epinephrine Hyperglycemia and Glycosuria.

Rabbit.		Amount of substance injected per kilo of body weight.		Blood sugar content (percentage).		Urine (in $7\frac{1}{2}$ hrs.).		
No.	Body weight.	Epinephrine.	Gelatin, egg albumin, or soluble starch.	$1\frac{1}{2}$ hrs. after injection.	3 hrs. after injection.	Volume.	Sugar.	
Gelatin.								
	kg.	*mg.*	*gm.*			*cc.*	*per cent*	*gm.*
I	1.66	0.75	0.2		0.21	37	0	0
II	1.46	1.0	0.15	0.31	0.34	59	0.77	0.45
III	2.24	1.0	0.15	0.31	0.32	131	0.85	1.11
Egg albumin.								
IV	1.68	1.0	0.15	0.29	0.37	35	1.03	0.36
V	1.70	1.0	0.075	0.28	0.31	109	0.63	0.69
Soluble starch.								
VI	2.54		0.5	0.11	0.10	109	0	0
VII	2.36	1.0	0.25	0.41	0.39	104	1.51	1.57
VIII	2.28	1.0	0.5	0.37	0.37	86	3.53	3.04

cosuria was distinctly decreased by them. Soluble starch seems to have no marked influence upon either hyperglycemia or glycosuria.

When soluble starch was injected without epinephrine (Rabbit VI in Table VI), the sugar content of the blood showed a normal value. The urine, collected $7\frac{1}{2}$ hours after the injection, contained no reducing sugar, but showed a strong dextrorotation. When Mendel and Mitchell injected soluble starch intraperitoneally into a dog and a rabbit, they found a dextrorotatory substance in the urine, and considered this substance as the injected carbohydrate altered only slightly, if at all. Owing to the presence of such a dextrorotatory substance, which cannot be removed by mercuric acetate, the urinary sugar of the starch experiments was estimated by Pflüger's gravimetric method.

Ellinger and Seelig demonstrated that severe bacterial infection

and cantharidine nephritis markedly diminish epinephrine glycosuria in rabbits. Under these circumstances the function of the kidneys was severely affected, the urine containing a large amount of blood or protein, and the volume of the urine being distinctly diminished.

Injecting Witte's peptone and egg white subcutaneously into rabbits, de Waele and Vandevelde studied the fate of the injected substances. Witte's peptone was injected in doses of 0.2 to 1.0 gm., egg white in doses of 10 to 25 cc. The function of the kidneys was not markedly impaired. On the whole, the difference between the urine volume and nitrogen elimination, before and after the injection, was very slight. When peptone was injected, traces of albumin or peptone were sometimes found in the urine. After the injection of egg white, a small amount of protein and also sometimes a trace of peptone were demonstrated in the urine. Though the test of the renal function by nitrogen or chloride estimation was not made in my present work, it is obvious that the renal permeability for sugar is distinctly decreased by intravenous injection of gelatin and egg albumin, notwithstanding that the urinary flow was not always affected. It is interesting to note that soluble starch, which has a colloidal character as well as peptone, gelatin, and egg albumin, behaves differently in this respect.

SUMMARY.

The blood sugar content of rabbits shows a tendency to increase after intravenous injection of Witte's peptone in doses of 0.5 to 0.75 gm. per kilo of body weight.

When hyperglycemia obtains, it continues for a few hours only.

In no case was there any evidence of a hypoglycemia alleged by McGuigan and Ross to be induced in dogs by peptone injection.

In the present investigation no difference could be observed upon the blood sugar content between boiled and unboiled peptone solution.

The intravenous injection of gelatin or egg albumin has little or no influence upon the sugar content of the blood.

Although an intravenous injection of peptone may be without marked influence upon epinephrine hyperglycemia, glycosuria

is distinctly diminished. The influence of peptone is ascribed to a possible change in renal permeability for sugar.

The influence of intravenous injection of gelatin and egg albumin upon epinephrine hyperglycemia and glycosuria is apparently the same as that described for peptone.

The fatal results experienced with a combination of protein and epinephrine injections cannot be ascribed to the colloidal character of the proteins, since soluble starch is without any detrimental influence under similar experimental conditions. Soluble starch exerts no action upon blood sugar content of normal rabbits or upon epinephrine hyperglycemia and glycosuria.

I desire to express my thanks to Professor Frank P. Underhill for his suggestions, help, and criticism, and also to Professor Lafayette B. Mendel for his advice.

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VARIATION IN THE AMOUNT OF PHOSPHATIDES IN THE CORPUS LUTEUM OF THE SOW DURING PREGNANCY.*

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Morphological studies of the corpus luteum have led several investigators to the view that the lutein cells contain considerable amounts of lipoids. Chauffard, Laroche, and Grigaut,[1] for instance, have laid emphasis upon the cholesterol content of the organ, Cesa-Bianchi[2] and others upon the lecithin or upon lipoid bodies of a less definitely indicated nature. A direct chemical confirmation of these results has been given by the recent analyses of Fenger[3] who finds that the corpora lutea of pregnant cows contain about thirteen times as great an amount of petroleum ether-soluble phosphorus as lean muscle, which he took as a standard of comparison.

The author has been investigating the microchemical reactions of the lutein cells of swine, and has also concluded that these cells contain an unusual amount of a lipoid or mixture of lipoids of the phosphatide group. In this species, the treatment

* An account of the cytological side of the work will appear in the *Anatomical Record* at an early date. The writer wishes to express his thanks to Professor T. Brailsford Robertson for advice on matters of biochemistry, and for the privileges of his laboratory; and to Mr. Ralston B. Brown, Superintendent of the Oakland Meat Company, for provision of fresh tissues.

[1] Chauffard, A., Laroche, G., and Grigaut, A., Fonction cholestérinigénique du corps jaune, *Compt. rend. Soc. biol.*, 1912, lxxii, 223, 265.

[2] Cesa-Bianchi, D., Di alcune particolarità di strutturi e dei fenomeni di secrezione del corpo luteo, *Internat. Monatschr. Anat. u. Physiol.*, 1908, xxv, 1.

[3] Fenger, F., Phosphatides in the ductless glands, *J. Biol. Chem.*, 1916, xxvii, 303.

of the corpus luteum with slow aqueous fixing fluids causes the rounding up into droplets of a substance which during the life of the tissue is diffusely present in the periphery of the cells. The droplets thus caused to appear are soluble in alcohol of 60 per cent or stronger, in ether, chloroform, acetone, benzene, and xylene; they stain blue with Nile blue sulfate, pale brick red with neutral red, yellowish with osmium tetroxide (changing to gray in alcohol), are positive with Ciaccio's method, and give a brown lake with Weigert's hematoxylin; they are not anisotropic.

Alcoholic extracts of the corpora lutea of swine contain, mixed with neutral fat, a fat-like substance of pasty consistence which is yellowish in color, readily becoming brown in the air, and presenting a characteristic "greasy" odor. It gives microchemical reactions exactly like those mentioned above for the droplets in the cells, except that as it rounds into spheres when in contact with water, the spheres are momentarily anisotropic.

The tests cited indicate that the substance, thus revealed by a fortunate artifact of fixation, is probably a lipoid or lipoid mixture of the phosphatide series, containing perhaps a fatty acid (suggested by the partial reduction of osmium tetroxide). Although Fenger has shown the presence of large amounts of phosphatides in the corpora lutea of cows, the microscopical appearances described above cannot be found in this species. Perhaps the lipoids are here in a less oily condition, not rounding up in water so readily; or they are dissolved in other cell lipoids which themselves do not produce the peculiar spheres which have been mentioned. In the dog the appearances are exactly as in swine, and certain bodies seen by Cohn[4] in the lutein cells of rabbits are almost certainly due to the same cause.

But the point of greatest interest is that the lipoid made visible by this method is present only during the earlier part of pregnancy, and cannot be detected after about the middle of the term. Chemical estimations of the alcohol-soluble phosphorus in the corpora lutea of swine also show a decrease in the amounts of the phosphatides in the advancing stages of pregnancy. In order to determine this point, the ovaries of sows at desired stages were removed immediately after slaughtering, and the corpora lutea

[4] Cohn, F., Zur Histologie und Histogenese des Corpus luteum und des interstitiellen Ovarialgewebes, *Arch. mikr. Anat.*, 1903, lxii, 745.

were shelled out of their capsules. The substance was desiccated by grinding in a mortar with three times their quantity by weight of a mixture of equal parts of anhydrous sodium and calcium sulfates, and drying the resultant paste to a powder over a water bath (Leathes[5]). The coarse powder was more finely pulverized and extracted with absolute alcohol in the Soxhlet apparatus for 3 days. Portions of the extract representing 5 gm. of the fresh gland were examined for phosphorus by von Wendt's modification of Neumann's method.[6] In the following table the phosphorus is expressed as P_2O_5, the figures therefore giving a relative indication of the amount of alcohol-soluble phosphorus-containing substances at the given stages.

	P_2O_5 per gm. of fresh tissue. *mg.*
Embryos 7 to 13 mm. long (early pregnancy)	0.63
Fetuses 98 to 140 mm. long (middle pregnancy)	0.43
Fetuses 190 to 270 mm. long (late pregnancy)	0.39

It cannot be too strongly emphasized that the corpus luteum is a very rapidly changing tissue. From the day of its sudden appearance until its atrophy, perhaps months later, its morphological appearance is in constant flux, and chemical studies must in the end be made with corpora lutea collected at known stages of the reproductive cycle, if they are to throw any light upon the physiology of the gland. The question naturally arises as to the rôle of the lipoids of the corpus. Robertson[7] has shown that the bodies of young animals contain a much higher percentage of alcohol-soluble phosphorus than those of the old. The corpus luteum of early pregnancy is, in effect, an islet of juvenile tissue among the older cells of its host. Is the larger amount of phospholipins, at this stage, merely an accompaniment of the youthfulness of the organ, or is it, as Fenger suggests, connected with the special secretory activities of the cells? The question offers possibilities of experimental test.

[5] Leathes, J. B., The Fats, London, 1910.

[6] Von Wendt, G., Untersuchung über den Eiweiss- und Salz-Stoffwechsel beim Menschen, *Skandin. Arch. Physiol.*, 1905, xvii, 211.

[7] Robertson, T. B., Experimental studies on growth. II. The normal growth of the white mouse, *J. Biol. Chem.*, 1916, xxiv, 380.

THE VITAMINE CONTENT OF BREWERS' YEAST.

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On account of the relation between the consumption of polished rice and the disease beri-beri, the early conceptions of vitamine were for the most part obtained from studies of rice polishings. It was soon shown, however, that curative fractions could be obtained from other sources. Among these ordinary brewers' yeast was found to be relatively rich in vitamine, and since it is a product which has so far not been extensively utilized in other ways, it may be considered a favorable raw material for vitamine studies.

Bottom yeast remaining after fermentation of the beer consists of a thick cream-colored mush. On warming, it rapidly expands with the evolution of carbon dioxide. The residual beer in it can be readily removed by pressing in a hydraulic or other suitable press. The amount of liquid thus removed is equal to about one-third to one-half of the weight of the original mass. The press cake when dried in a rapid current of air yields brownish brittle lumps which, if kept in a dry place, apparently remain unchanged for long periods. If the fresh press cake is warmed to a temperature of about 37.5° C. for 24 to 48 hours, it autolyzes more or less completely and yields a thick slimy liquid. This latter can be filtered only with great difficulty. A clear filtrate can best be obtained from it on a laboratory scale by use of large glass funnels and folded filter papers. It has been found that the yield of clear red-brown filtrate corresponds usually to about one-half the weight of the thick unfiltered liquid. The residue on the filter no doubt retains a considerable amount of the vitamine, but so far a satisfactory method for making a more complete separation of the liquid and undigested solids has not been found. If autolyzed yeast filtrate is kept in a cool place, it apparently remains unaltered for several months, except for the gradual deposition of a sediment, which appears to be greater the lower the temperature.

Activity of Autolyzed Yeast Filtrate.—In order to ascertain what quantity of autolyzed yeast filtrate is required to counter-

balance the deficiency of an exclusive diet of polished rice, the following feeding experiments were made on pigeons. The results are shown in Charts 1 and 3.[1] For the sake of comparison, the data for a large number of control pigeons fed on polished rice at various times during the course of the investigation are given in Chart 2.

Briefly, the experiments were made in all cases as follows: Groups of pigeons were kept in cages and given only water and polished rice. Each pigeon was weighed every 2nd or 3rd day, and the designated amount of the yeast filtrate administered with the aid of a small rubber tube. The figures for the abscissæ in the charts show the percentage change in weight as found by dividing the observed weight from day to day by the average weight of the bird for several days just preceding the experiment. The ordinates show the days the experiments were continued. From these charts it is seen that whereas control birds (Chart 2) receiving only polished rice decline to about 65 to 75 per cent of their original weight and develop typical polyneuritis within about 15 to 30 days, the birds which received doses of as little as 1.0 cc. of yeast filtrate (Chart 1) on alternate days lived for at least 3 months with, in two cases, no significant change in weight, and in a third a loss of approximately 25 per cent of its original weight, without showing symptoms of polyneuritis. The birds receiving 3 cc. doses of the yeast filtrate, except in one case which was probably a young growing bird, gained practically no more in weight than the birds receiving 1 cc. doses of the filtrate. It is probable, therefore, that more than the minimum amount of vitamine required to maintain normal weight is not utilized in the organism.

In the case of the bird which received 0.5 cc. of the autolyzed yeast filtrate on alternate days (Chart 1), a marked loss in weight occurred. Unfortunately this bird died on the 43rd day, probably of some other cause than polyneuritis. The bird which received 0.3 cc. of the filtrate on alternate days proceeded in the typical manner to the development of polyneuritis on the 38th day.

[1] The data for Chart 3 were kindly furnished by Mr. R. R. Williams, of the Bureau of Chemistry.

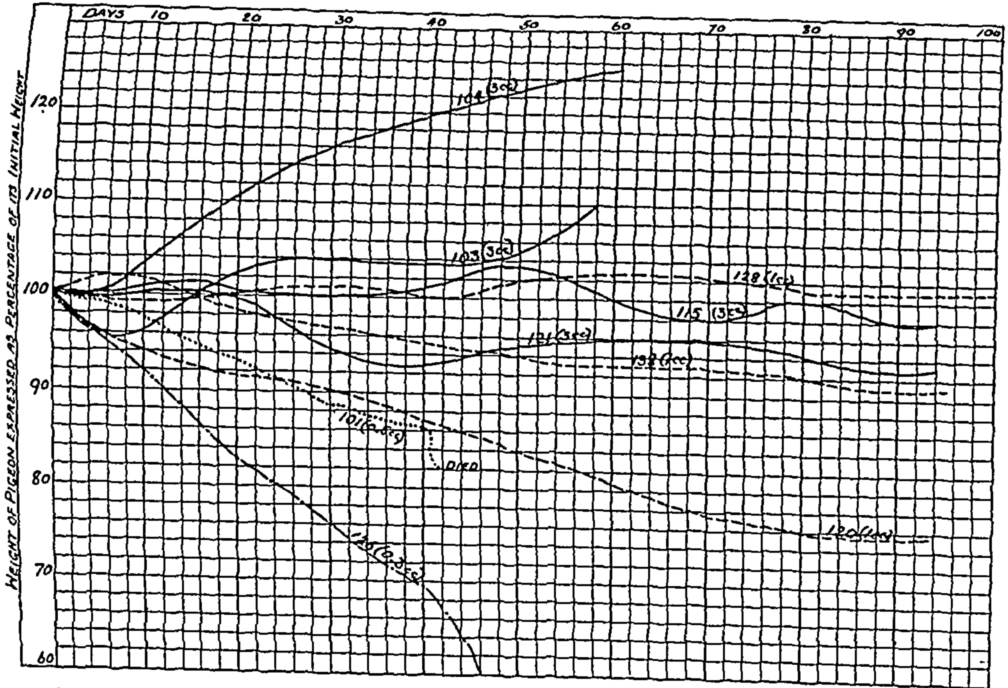

CHART 1. The change in weight of pigeons fed on polished rice and given the indicated quantities of autolyzed yeast filtrate on alternate days. The termination of the line, except for Pigeons 101 and 126 which died as shown, indicates the discontinuation of the experiment. The actual weighings give more or less irregular points (as shown in Chart 3) due to the large quantities of food which pigeons may take into their crops at irregular intervals.

Considering the results shown in Chart 3 it is seen that 1.0 cc. doses of the sample of yeast filtrate here used failed to protect the birds against a rapid loss in weight. This sample had been

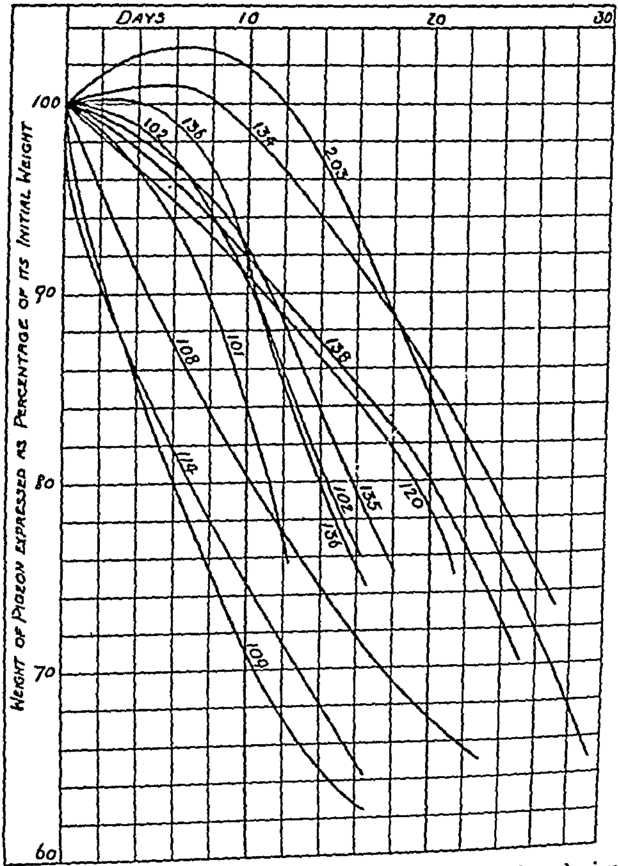

CHART 2. The rates of decline in weight of pigeons fed exclusively on polished rice. The termination of the lines indicates development of typical polyneuritic paralysis or death of the pigeon. The curves represent the average loss in weight. The actual points show irregularities due to daily variation in quantity of food eaten by the pigeon.

kept in a cool place for several months preceding its use and may have suffered some deterioration or may possibly have contained less vitamine in the first place than was present in the filtrate used in the experiment recorded in *Chart 1*. It is of

interest to note that an increase of the dose to 2.0 cc. soon overcame the deficiency and caused an increase in the weight of the birds. This experiment appears to indicate that a comparatively slight deficiency from the minimum daily vitamine requirements will result in a perceptible loss in weight, and the restoration of this deficiency will be eventually followed by an increase in the weight.

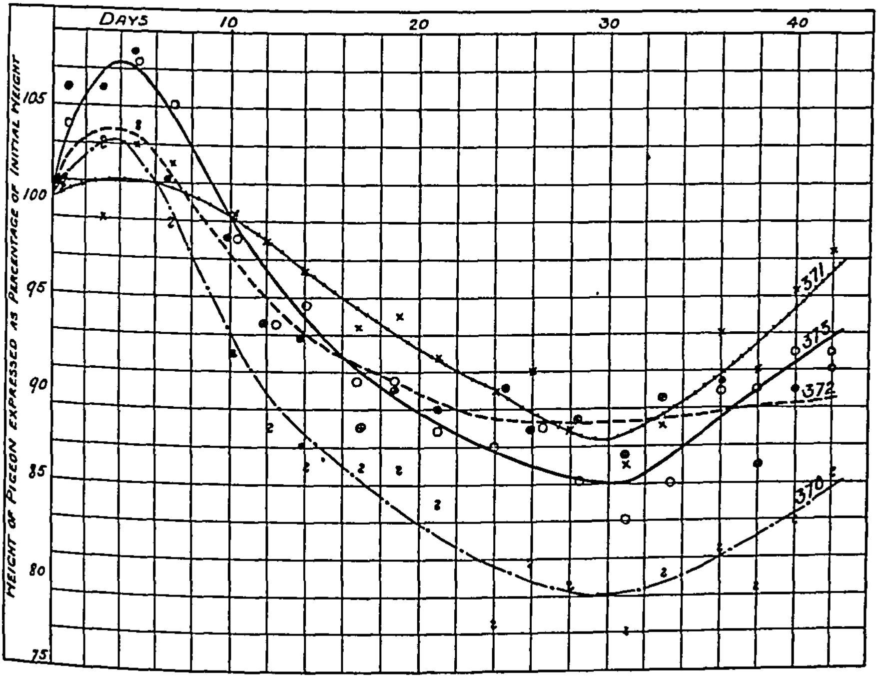

CHART 3. The change in weight of pigeons fed on polished rice and given doses of autolyzed yeast filtrate on alternate days. Each bird received 1.0 cc. doses from the 1st to the 17th day and 2.0 cc. doses thereafter. The actual weighings are indicated on the chart. The lines are intended to represent the average of these points.

From these results it is apparent that less than 1.0 cc. of autolyzed yeast filtrate given on alternate days to a pigeon fed only on polished rice is insufficient to prevent loss in weight and eventual development of polyneuritis. This quantity may in some cases be less than enough to prevent loss in weight, and in others be entirely adequate. It is, therefore, probable that the quan-

tity of vitamine in 0.5 cc. of filtrate from autolyzed pressed brewers' yeast, freshly prepared as described above, is just sufficient to supply the daily vitamine requirement of a grown pigeon of average size.

Activity of Dried Yeast.—In order to compare the activity of dried yeast with that of the autolyzed material, a feeding experiment similar to the above was made with several samples of dried yeast. Of these, one was prepared in the laboratory by simply blowing a rapid current of air over a thin layer of freshly pressed brewers' yeast for about 24 hours or until it was dry and brittle. Two of the samples were kindly prepared by the Wittemann Company of Buffalo, with the aid of an apparatus consisting of two steam heated rotating drums (steam pressure averaging about 35 pounds per square inch) between which the yeast passes and from which the dried material is scraped after being in contact with the heated drums for approximately 40 seconds. Of these two samples, one was prepared from the fresh brewers' yeast just as obtained from the brewery, the other was made from brewers' yeast which was first allowed to autolyze in a warm place for 48 hours previous to being put through the drying apparatus. The samples described above were designated, for convenience, laboratory dried yeast, Wittemann dried fresh yeast, and Wittemann dried autolyzed yeast.

The results of the feeding experiment on pigeons made with these three samples are shown in Chart 4. The dose of the dried products selected for this experiment was estimated to be approximately equivalent to the quantity, *viz.*, 1.0 cc. of clear autolyzed yeast filtrate, which, as shown in Chart 1, appeared to be just sufficient to replace the deficiency of a polished rice diet. Total solids determinations on autolyzed yeast filtrate (sp. gr. 1.06 at 15°C.) showed an average of about 0.2 gm. per 1.0 cc. It would be expected that the unfiltered autolyzed yeast (which of course is equivalent weight for weight to fresh pressed yeast) would contain approximately 0.25 gm. of dry residue per 1.0 cc. Direct determination on freshly pressed yeast showed a moisture content of about 75 per cent. It appears, therefore, reasonable to assume that the 0.25 gm. dose of dried yeast used in the experiment is approximately equivalent in potential vitamine to 1.0 cc. of autolyzed yeast filtrate.

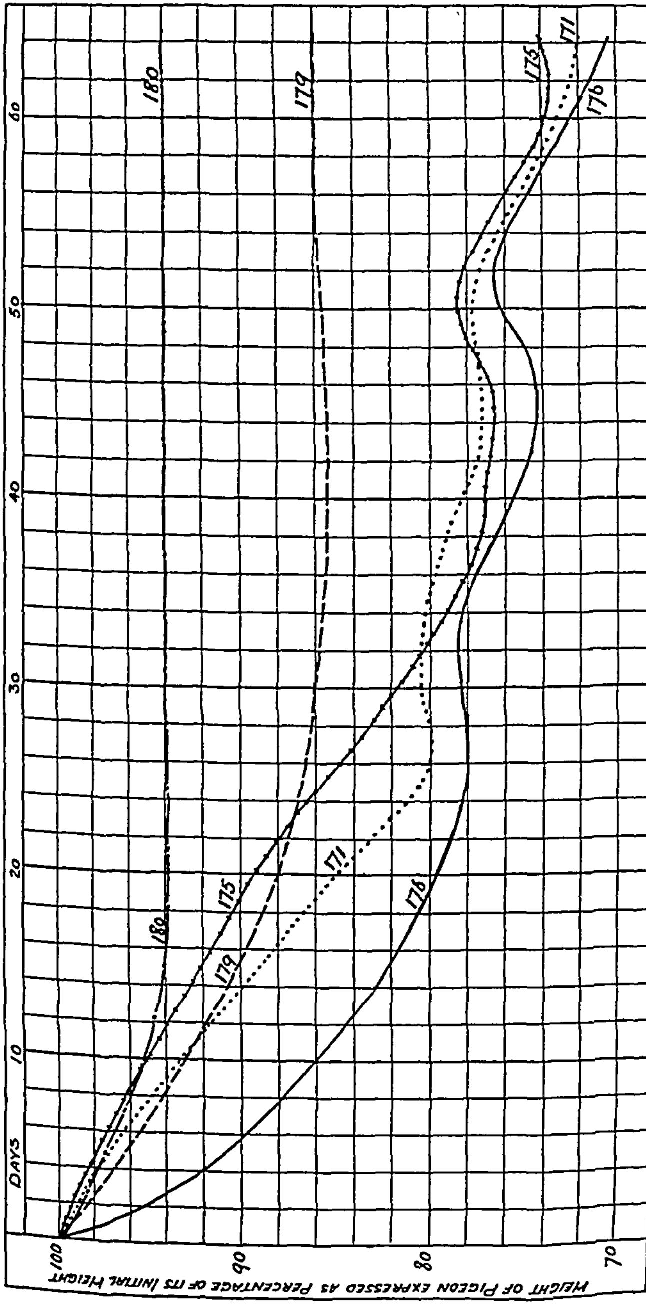

CHART 4. The change in weight of pigeons fed on polished rice and given 0.25 gm. doses of dried yeast on alternate days. Bird 171 received the laboratory dried product. Birds 175 and 176 received the Wittemann dried fresh yeast, and Birds 179 and 180 received the Wittemann dried *autolyzed* yeast.

On comparing the weight curves in Chart 4 with those in Chart 1, it is seen that considerably less protection was afforded by the dried products than was found for presumably equivalent doses of the clear liquid filtrate. It seems, therefore, probable that to replace a given diet deficiency somewhat larger quantities of dried yeast than of autolyzed yeast are required.

Influence of Autolysis upon Yeast Vitamine.—Considering further the results shown in Chart 4, it is seen that Birds 171, 175, and 176, which received the dried fresh yeast, lost considerably more weight on a rice diet than Birds 179 and 180, which received the dried autolyzed product. The only difference in the two cases, as already pointed out, was that one lot of the brewers' yeast was allowed to autolyze before being dried and the other was not. The experiment appears, therefore, to show conclusively that autolysis increases the protection afforded by the vitamine of brewers' yeast.

Additional evidence confirming this conclusion was obtained from another feeding experiment in which doses of 0.05 gm. of the dried products were used. This amount was far too small to afford more than a slight protection, but it is interesting to note that the two birds receiving the autolyzed product lost weight distinctly less rapidly than the two receiving the freshly dried material.

Estimation of the Quantity of Vitamine Isolated from Yeast Filtrate by Adsorption to Fullers' Earth.[2]—Since it has been shown by previous experiments[3] that fullers' earth selectively adsorbs vitamine from autolyzed yeast filtrate, it was thought that advantage of this property could be taken to separate the vitamine from the greater part of the other nitrogenous compounds which accompany it and obtain an approximate estimate of its quantity by determination of the nitrogen retained by the fullers' earth.

Samples of fullers' earth activated by being shaken with autolyzed yeast filtrate were found to contain approximately 2 per cent of nitrogen. The question arises, however, as to whether this nitrogen represents only vitamine or a mixture of vitamine and other nitrogenous compounds.

[2] The particular variety of fullers' earth so far used in this work has been generously supplied by Professor John U. Lloyd of Cincinnati, and is characterized by its exceptionally high adsorptive capacity for alkaloids.

[3] Seidell, A., *U. S. Pub. Health Reports*, 1916, xxxi, 364.

To obtain data on this point, 100 cc. of a sample of autolyzed yeast filtrate prepared as previously described, were shaken for several minutes with 5.0 gm. of fullers' earth, and the solid was removed by filtration. The clear liquid was then shaken with a second 5.0 gm. portion of the fullers' earth, the mixture filtered, and this procedure repeated a third time. Nitrogen determinations made on the original yeast filtrate and on each of the samples of fullers' earth which had been shaken in succession with the liquid, gave the following results. The original liquid contained 2.0 gm. of nitrogen per 100 cc. The first 5.0 gm. portion of fullers' earth contained 0.090 gm. of nitrogen, the second 0.073 gm., and the third 0.058 gm.

Experiments reported in the paper referred to above showed that with a ratio of 5 gm. of solid per 100 cc. of liquid, practically all the vitamine is removed from yeast filtrate. From the present results, which show that additional nitrogen is adsorbed by further treatment with fullers' earth, it is apparent that vitamine is only one of perhaps several nitrogenous compounds removable from yeast filtrate by fullers' earth. Although according to the previous experiments all the vitamine appears to be in the first fraction, it does not follow that other nitrogenous compounds are absent from this fraction. In fact it would be expected that some are adsorbed simultaneously with the vitamine. Therefore, the 0.09 gm. of nitrogen in the first lot of fullers' earth is probably representative of both vitamine and other nitrogenous compounds. How great a percentage of the latter is present can, of course, not be judged until further experiments are made. In the meantime it has been thought of interest to neglect the presence of any other nitrogenous compounds and assume that the nitrogen found represents only vitamine. In this way an approximate estimate of the maximum quantity of vitamine in yeast filtrate will be obtained.

From recent experiments by Williams and Seidell[4] it seems probable that vitamine contains approximately 50 per cent of nitrogen. On this basis, if it is assumed that all the 0.09 gm. of nitrogen in the first 5.0 gm. of fullers' earth shaken with 100 cc. of yeast filtrate as described above is vitamine nitrogen, it follows that 0.18 gm. per 100 cc. of autolyzed yeast filtrate is the maximum amount of vitamine which can be present.

[4] Williams, R. R., and Seidell, A., *J. Biol. Chem.*, 1916, xxvi, 431.

The feeding experiments on pigeons described in the preceding pages show that approximately 1.0 cc. of yeast filtrate given every other day is close to the amount required just to replace the deficiency of an exclusive diet of polished rice. Since this 1.0 cc. of yeast filtrate contains not exceeding 0.0018 gm. of vitamine, the daily vitamine requirement of pigeons must be somewhat less than 1 mg.

Recent observations have shown that pigeons given as much as they will eat consume about 30 gm. of food daily, per bird. If, as shown above, not more than 1 mg. of this must be vitamine, a properly balanced diet for a pigeon will be one which contains somewhat less than 0.0033 per cent of vitamine.

Although it cannot be stated with certainty, it is probable that a vitamine content of the same magnitude in the food of man would meet the requirements of the human organism.

SUMMARY.

a. In the case of pigeons, the vitamine deficiency of an exclusive diet of polished rice is just replaced by daily doses of 0.5 to 1.0 cc. of the clear filtrate from autolyzed brewers' yeast.

b. Doses of dried freshly pressed yeast approximately equivalent to 1.0 cc. doses of autolyzed yeast do not effectively replace the vitamine deficiency of a diet of polished rice.

c. Of two samples of dried yeast, one of which was autolyzed previous to being dried and the other not, the pigeons receiving the latter lost weight on a polished rice diet considerably more promptly than those receiving the former. The autolytic process therefore appears to influence favorably the activity of the vitamine of brewers' yeast.

d. On the assumption that all the nitrogen contained in fullers' earth which has been shaken with autolyzed yeast filtrate is derived from vitamine, the maximum quantity of the latter which can be present in the original yeast filtrate is 0.18 gm. per 100 cc.

e. The daily vitamine requirement of a grown pigeon is somewhat less than 1 mg. A diet containing 0.0033 per cent of vitamine, given in quantities ordinarily consumed by pigeons, will supply this requirement.

AN IMPROVED NEPHELOMETER-COLORIMETER.*

By PHILIP ADOLPH KOBER.

(*From the Division of Laboratories and Research, New York State Department of Health, Albany.*)

(Received for publication, January 4, 1917.)

CONTENTS.

INTRODUCTION.

In biological chemical research work it is sometimes necessary to build a special apparatus or improve or modify an old one. At the outbreak of the European war it was impossible to obtain Duboscq colorimeters, and since it was desirable to remedy some defects in its design, a new instrument was developed and built in this country.[1] The purpose of this paper is to discuss some essential points of nephelometers and colorimeters, and to show still further improvements in the design and construction of a nephelometer-colorimeter.[2]

* A preliminary note was read at the New York meeting of the American Chemical Society, before the Biological Section, September 29, 1916.

[1] Manufactured by Lenz and Naumann, Inc., New York.

[2] The new instrument is manufactured by Klett Manufacturing Company, Inc., 202 East 46th Street, New York, and may be obtained from them or through dealers. The price, including two cups of any kind, is about $54.00. The lamp house is quoted at $24.00; extra cups at $1.50 each; light filters at $3.00 a pair. The whole outfit, including eight cups (two of each kind), mounted on a suitable black board with light switch, ready for use, is priced at $90.00.

Discussion of Other Instruments.

Bloor Nephelometer.—In order to provide a suitable mechanical and optical equipment for the Richards type of instrument, Bloor[3] modified the Duboscq colorimeter by removing the plungers and adding a few simple and easily constructed parts. That this resulted in a decided improvement for that type of instrument cannot be doubted, but the original *criticisms*[4] *against the Richards instrument* are *still valid*; *i.e.*, sources of error due to variations in the meniscus and in the indirect reflection of light from suspended matter in the lower part of the tubes. Bloor's objections to the plunger type of nephelometer are, however, no longer valid. As mentioned elsewhere in this paper, the plunger is made of one piece of glass by fusion of black glass tubing with an optical glass end, and therefore has no paint which may dissolve or peel off. It can now be kept as free from contamination as any other glass object and if necessary can be easily removed and replaced or cleaned with the strongest acid cleaning solution, if so desired.

An apparent advantage of the Richards type is that the solutions are not stirred or agitated by the plungers while obtaining a photometric balance, if we assume that agitation tends to agglutinate the suspensions, and ignore the fact that agitation also tends to keep any agglutinated particles in the suspension from settling. That this advantage is more apparent than real is due not only to the fact that all liquids are considerably more agitated, and practically to the same extent no matter what instrument is used, when adding the suspensions to the nephelometric containers, but also to the fact that the agitation produced by moving a plunger slowly up and down while making a reading is so slight that it must be negligible in most cases. However, any advantage derived by freedom from agitation is wholly lost by sources of errors, introduced in this instrument elsewhere as pointed out above. The relative accuracy obtained with it when compared with other instruments under favorable and equal conditions is not known.

Marshall-Banks Nephelometer.[5]—This instrument is described as having "equal columns of suspensions," and "actually measuring the reflected lights with a suitable photometer," "the photometric part of the apparatus consists of a wedge[6] of neutral tinted glass," while the "optical arrangement for observing the two beams of light" consisted of "a simple

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[3] Bloor, W. R., *J. Biol. Chem.*, 1915, xxii, 145.

[4] Kober, P. A., *J. Biol. Chem.*, 1912–13, xiii, 485.

[5] Marshall, J. T. W., and Banks, H. W., 3rd, *Proc. Am. Phil. Soc.*, 1915. liv, 180.

[6] The smoked glass wedge was used for photometric work in astronomy in 1882 (see Pickering, E. C., Nature, 1882, xxvi, 259) but was not adopted by photometrists.

arrangement of mirrors[7] by which a field far more sensitive than that of a Duboscq colorimeter may be obtained." As a detailed discussion of this instrument may appear in a separate communication on the chemical work also reported by Marshall and Banks in the papers dealing with their instrument, nothing further will be added here.

Duboscq Colorimeter.—Folin[8] mentions a number of defects in the Duboscq colorimeter: (1) inaccuracy of the zero points; (2) liability to dust interference; (3) inequality of illumination in the fields; (4) another disadvantage develops when the variations in the amount of color in the liquids to be measured are great, in which case two different and expensive colorimeters are necessary; (5) as the Duboscq cups have cemented bottoms they cannot be used for all solvents, and, as an investigator recently found, it was necessary to change the cement for certain work; (6) when the colorimeter is to be adapted as a nephelometer other disadvantages become apparent, especially in connection with the plungers and cups. This was pointed out in a previous paper.[1?]

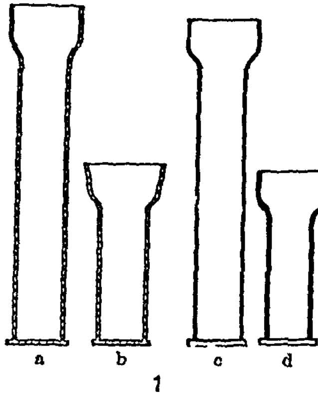
Lenzman-Kober Nephelometer-Colorimeter.[9]—This instrument practically eliminates the defects mentioned in the Duboscq colorimeter, with exception of defect (3), inequality of field illumination. This is however removed by means of the adjustable reflectors of the new lamp house (page 162). (1) In the Lenzman-Kober instrument one or two adjustments of the adjustable verniers eliminate any inaccuracy of the zero point; (2) no open spaces are to be found between the top of the eye piece and the liquids in the cups so that dust is excluded; (3) the scale and instrument are also so constructed that any height of liquid up to 110 mm. can be measured; (4) the colorimetric cups (Fig. 1) in this instrument, like the new nephelometer cups (Fig. 2) with which they are interchangeable, are fused instead of cemented and are therefore of one piece of glass, usable for all solvents. (5) The black glass plunger[10] (Fig. 3) with fused-

[7] A similar arrangement of mirrors is used in the Donnan-Köhler colorimeter (described in *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, i, 648), which arrangement, owing to the loss of light by reflection of the glass of the front mirror, and owing to the unequal paths the light from the cups must travel, requires, as stated in the book, an empirical correction.

[8] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 484.

[9] Made by Lenz and Naumann, Inc., New York, at the suggestion and with the cooperation of the author.

[10] For this idea and for much experimental work at his own expense, I am indebted to Mr. Hugo Schnackenberg, of the firm of Potter and Schnackenberg, Manufacturing Opticians, New York.



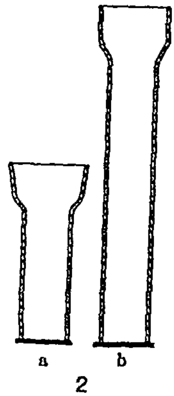


FIG. 1. Colorimetric cups. (*a, b*) Transparent and optically clear bottoms fused to the sides; (c, *d*) black glass sides which eliminate the colorimetric light shield.

FIG. 2. Nephelometric cups. (*a, b*) Black glass bottoms fused to trans-t sides. Diameter of cups just ½ mm. larger than that of the plungers.

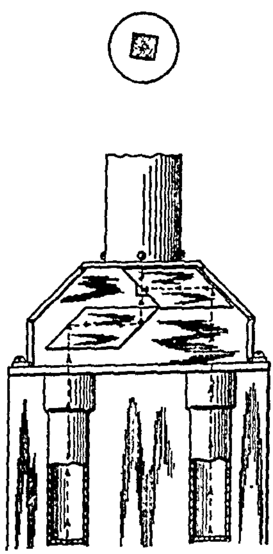


FIG. 3. Prism arrangement and plungers. Black glass plungers with optically clear glass ends, fused together. Prism arrangement, which gives a field similar to that of Lummer-Brodhun.

in, optically clear bottoms, not cemented, as they might seem and a few have believed them to be, makes its use as a colorimeter and nephelometer a matter of equal ease and accuracy, entirely eliminating the use of asphaltum paint for this purpose. The best Duboscq plungers show a distinct color or absorption of light when compared with these new plungers. (6) The new design of a field (Fig. 3) similiar to that of Lummer-Brodhun, a square within a circle, makes its use, when correctly made and adjusted, much more comfortable for the eye and more sensitive.

Unfortunately the first lot of twenty-five instruments, owing to the difficulties arising from the scarcity of good optical glass, superimposed upon labor difficulties, were imperfectly and crudely made, poorly if at all adjusted, so that the advantages of the new design were rarely apparent. In the second lot of twenty-five and subsequent instruments, the difficulties seem to have been completely overcome.

This instrument for its use as a nephelometer, however, had a disadvantage in that it used automatic black cloth curtains necessary to insure a black background for the opalescent solutions. In common with the Duboscq colorimeter, it had also racks and pinions for raising the cups or plungers, which are difficult to make and almost impossible to use without having appreciable lost motion.

The New Instrument.

The Nephelometer-Colorimeter.—By using a screw arrangement,[11] as shown in Figs. 4 and 5, both the black curtains and this lost motion can be eliminated and the construction considerably simplified. As may be seen, it also has all the advantages of the Lenzman-Kober instrument mentioned before. Furthermore, with the double milled head, a double adjustment is practically secured: (1) with the small diameter, a quick action for rough adjustment; (2) with the large diameter, a slow action, for fine adjustment. Another advantage in construction is seen in the scale, which shows that it is removable, so

[11] For this idea and for much experimental work at his own expense, I am indebted to Mr. R. E. Klett, of the Klett Manufacturing Company, Inc., New York.

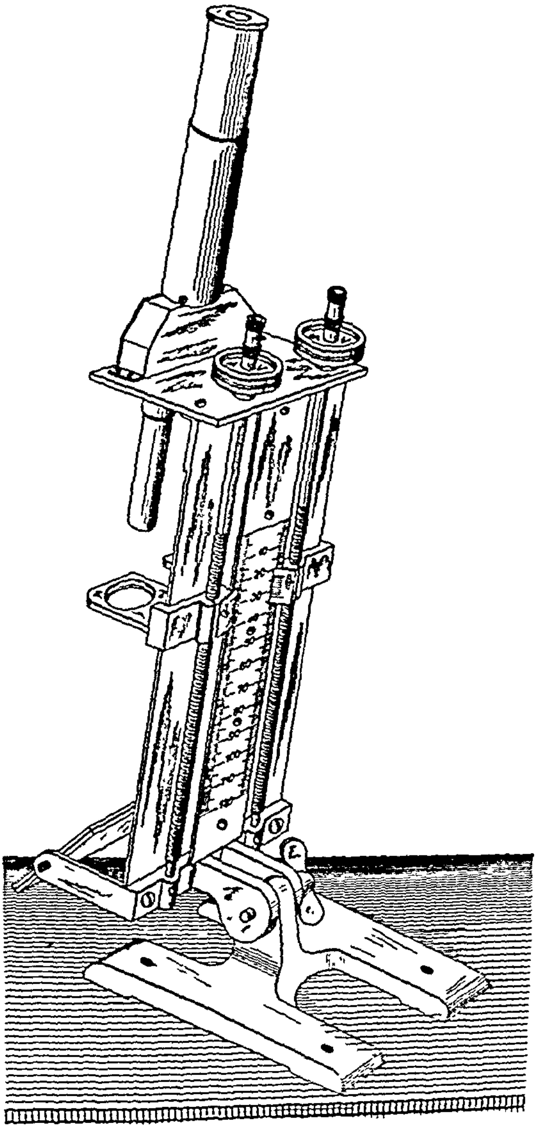


FIG. 4. The new nephelometer-colorimeter, showing screw arrangement with adjustable verniers, also the double milled head. There are no open spaces through which dust may enter and light escape when fitted to the lamp house (see Fig. 5).

that it can be repaired or replaced by a new one when necessary. As these scales are made with a dividing machine, they are usually very exact.

The Lamp House.—In a previous communication[12] the theoretical qualifications of a nephelometric light were discussed. The lack of a convenient source of low voltage (from 6 to 20

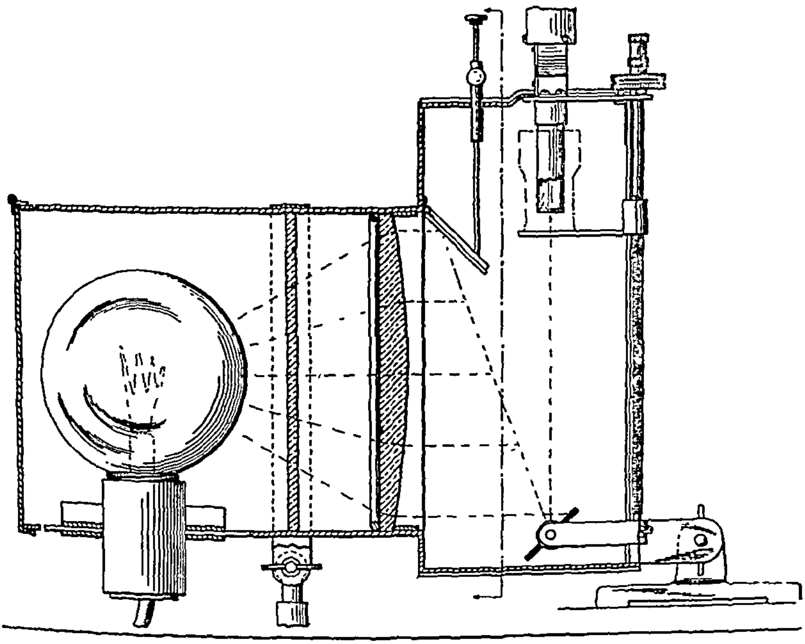


FIG. 5. Lamp house and instrument, showing the concentrated filament lamp, air space, condenser, and lamp house. When the doors (not shown) are closed, no light is visible except in the eye piece. The inclined angle of the instrument, which allows air bubbles to escape from underneath the plungers, and the exact position and angle of reflectors are not shown in the sketch.

volts) makes it still difficult if not impossible to obtain parallel rays of light, without using an arc lamp. The arrangement, as shown in Fig. 5, of a concentrated tungsten filament 110–120

[12] Kober, P. A., and Graves, S. S., *J. Ind. and Eng. Chem.*, 1915, vii, 843.

volt lamp[13] (stereopticon) with a condenser gives a very strong light, suitable for most work. A pane of glass is interposed between it and the condenser, so as to provide an air space, to prevent unnecessary heating of condenser and nephelometer. The lamp house can then be directly connected to most lighting circuits. The reflectors (Fig. 6) of the lamp house are made in pairs, so that the light which falls on each side of the colorimeter

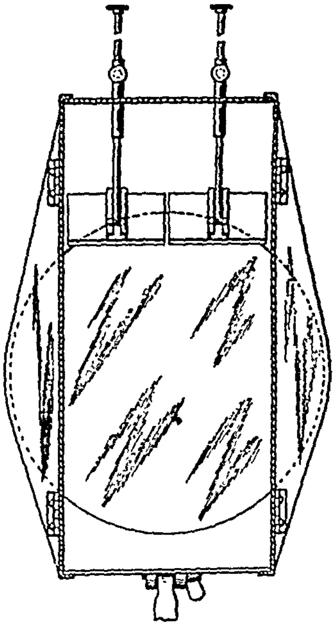


FIG. 6. Colorimetric reflectors, showing two separate reflectors by means of which the light reaching the eye piece may be adjusted to equality.

can be adjusted to produce equality in the field, thus eliminating a defect in the Duboscq colorimeter mentioned by Folin. This defect, which is more or less present in all these instruments, is due to the fact that it is impossible to make optical and mechanical parts perfectly.[14] The individual reflectors once adjusted

[13] McDermott, F. A., *J. Am. Chem. Soc.*, 1914, xxxvi, 454, describes a lamp of the same construction.

[14] In one of the most accurate of physical instruments, the balance, equality of sides, or the zero point, often changes and is also not adjusted until it is set up ready for use.

and the position marked on the stem, they can be removed from the field of light for nephelometric work and replaced if necessary in a moment. In Fig. 7 is shown a removable light filter, which can be made of this so called "daylight" glass if its absorption of light is not too great, or a light blue glass to remove any yellow in the artificial light, so as to measure any yellowish liquids. This allows the instrument to be set up perma-

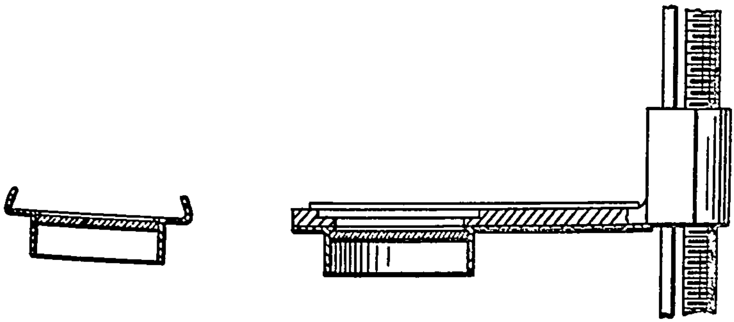


Fig. 7. Removable light filters. These filters, being attached underneath the cup holders, may remain on the instrument without interfering with nephelometric work.

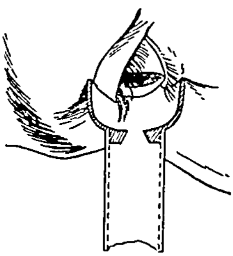


Fig. 8. Eye support which steadies the eye and keeps it at one point.

nently, screwed to a base, so as to be portable, and yet equipped to measure quantitatively colored and opalescent liquids with equal facility and almost equal accuracy. There is a growing conviction that in general it is easier to measure nephelometrically than colorimetrically, due to the fact that the more light absorbed by the solution, *i.e.*, the more it approaches uniform absorption, the easier it is to measure. For example, a blue or a

green[15] tint is easier to match than a yellow tint. In Fig. 8 is shown an eye support which will be of assistance in keeping the eye steady and always at the same point, an important requisite for precision work.

Results with the New Instrument.

The following readings made on the first factory model, by an assistant who had practically no photometric experience, show not only the sensitiveness of the instrument, but also the accuracy obtainable in nephelometric work.

Casein with Sulfosalicylic Acid.

Solution I.	Solution II.	Solution III.	Solution III.
16.3	24.9	30.3	30.2
16.5	24.6	30.5	30.4
16.2	24.7	30.2	30.3
16.3	24.5	30.3	30.4
16.33	24.67	30.33	30.33

As the new instrument has stationary plungers and movable cups, while most of the previous nephelometric work was done with movable plungers and stationary cups, it was important to run a nephelometric curve to see the effect on the constants. The curve shown in Fig. 9, with casein and sulfosalicylic acid, shows that in these new instruments the nephelometric constant has a new value and sign.

[15] Elbert, *Wiedemann's Ann.*, xxxiii, 136, found the following relative values of stimulus threshold for one individual.

	Wave length. μ	Threshold values.
Red	0.675	0.7
Yellow	0.590	2.2
Green	0.530	0.5
Bluish green	0.500	1.0
Blue	0.470	7.0

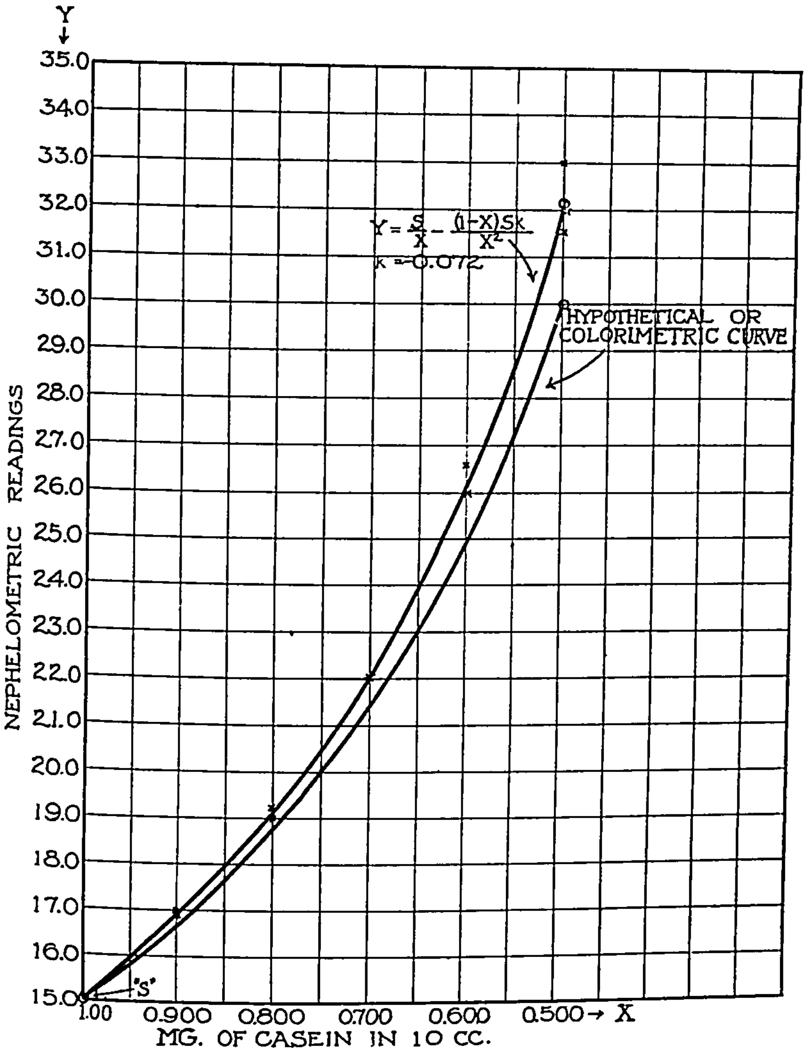


FIG. 9. Curve, with the new instrument, using casein.

Ratio of solutions. x	Actual readings.			Average readings. y	$y = \frac{s}{x} - \frac{(1-x)\,s\,k}{x^2}$ $k = -0.072$
	mm.			mm.	
1.0	15.0	15.0	= s	15.00	15.00
0.9	16.9	17.0	16.8	16.90	16.80
0.8	18.9	18.9	19.2	19.00	19.09
0.7	22.00	22.00		22.00	22.07
0.6	26.0	26.5		26.25	26.20
0.5	31.50	32.0	33.0*	32.17	32.16

* This curve was made quickly at the close of a day (after 9.30 p.m.) and the last readings show the effect of eye fatigue.

With casein, using the old type of instrument where the light from the weaker solutions had to travel a mean distance that was horter than the mean path of the stronger solution, the constant was about + 0.20, whereas in this new instrument where the light from the stronger solution has shorter mean distance to travel, the constant is − 0.072.

From this we learn that part of the deviation from the hypothetical or colorimetric curve was due to differences in the mean paths, while the other part of the deviation must be due to the effect of the suspension on the amount of light transmitted.[16]

While it is to be remembered that this formula expresses only the ratio of the solutions and is not for absolute photometry, it is, nevertheless, remarkable that so simple and empirical a formula is capable of expressing with precision a phenomenon which has so many factors.

With the same instrument as a colorimeter, measuring cupric sulfate and potassium dichromate solutions, the following readings were obtained.

	$CuSO_4$	$K_2Cr_2O_7$
	21.0	19.9
	20.8	19.5
	20.7	19.6
	20.8	19.6
	21.1	19.9
Average	20.9	19.7

[16] Discussion of this and other aspects of nephelometry is reserved for a subsequent publication. It may be possible to obtain a formula having a constant, independent of the type of instrument.

Directions for Using the Instrument.

In previous papers[17] the use of nephelometric curves (Fig. 9) and a formula was discussed and it was pointed out that it is necessary to standardize the instrument, using solutions of different concentrations. When the instrument changed so that a restandardization was necessary, a nephelometric formula obviated considerable work, especially the readings for and the drawing of a new curve.

Since the formula is complicated and many prefer to do without mathematical calculations, the following scheme can be used. The instrument is standardized as before, but the curve (see Fig. 9) is used alone in getting the amount of substance equivalent to the readings. When the value of the standard readings (designated by *s* in the formula) changes, the height of the solution used as a tare is adjusted so that the original reading for *s* is obtained, and therefore the original curve is applicable. It is equivalent to changing the zero point of a balance by adjustment so as to avoid calculations.

It is doubtful, however, whether the use of such a curve can ever be as accurate as use of the formula, owing to the fact that the accuracy with which one can draw and use a curve is decidedly limited, while the accuracy with which one can express relationships by means of an equation is unlimited.

A number of inquiries have been received as to obtaining the correct zero reading with the verniers, when the cups are too long to permit the plunger to touch the bottom of the cups, *i.e.*, to obtain the exact position on the scale for zero light or color. A number of methods could be suggested but the following is probably the simplest. A piece of wood, such as an ordinary cylindrical or hexagonal lead pencil or a thick glass rod or tube, about the thickness of the plunger, is cut off about 70 mm. long, making sure that the ends are parallel and at right angles to the sides. The length of this is then accurately measured with the scale on the instrument, or any other accurate mm. scale. This meas-

[17] Kober, *J. Biol. Chem.*, 1912–13, xiii, 485. Kober, P. A., and Egerer, G., *J. Am. Chem. Soc.*, 1915, xxxvii, 2377 (also given in Hawk, P. B., Practical Physiological Chemistry, Philadelphia, 5th edition, 1916, 294).

ured rod[18] is put into the cup and the instrument adjusted until the plunger touches it, or is flush with its optical end. If the screw of the vernier is loosened and the vernier set on scale to correspond with the length of the rod then the vernier and scale will give the actual height of space or liquid underneath the plunger in actual work. It is not safe to assume that all the cups have bottoms of the same thickness.

SUMMARY.

1. A new nephelometer-colorimeter has been described which has the following advantages: (*a*) a screw arrangement for changing the heights of liquids, and therefore the elimination of lost motion inherent in racks and pinions; (*b*) the elimination of dark cloth curtains; (*c*) black one piece glass plungers; (*d*) fused one piece nephelometric and colorimetric cups; (*e*) a convenient eye support.

2. A simple and convenient lamp and lamp house are described, having adjustable reflectors which permit the amount of light reaching both sides of the colorimetric field to be adjusted to equality.

3. A new simplified method of using the instrument is given.

[18] An accurately measured aluminium rod called "zero-gauge" can be obtained from Klett Manufacturing Company, Inc., New York.

A RAPID METHOD FOR DETERMINING CALCIUM IN BLOOD AND MILK.

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(Received for publication, January 4, 1917.)

In 1915[1] I published a short turbidity method for determining calcium in feces and urine, which consisted briefly of the following steps. The calcium was precipitated as calcium oxalate in the urine (or the acid extract of fecal ash) under conditions to minimize the occlusion of magnesium; the precipitate was centrifuged, washed, dissolved in dilute hydrochloric acid, and treated with an aqueous solution of the potassium soap of castor oil. The resulting cloud of calcium soap was read on the Duboscq colorimeter against a standard solution of calcium oxalate in hydrochloric acid, treated with the soap at the same time as the unknown.

I have now succeeded in applying essentially these same principles to the determination of calcium in small amounts of blood and of milk, the two chief differences being, first, that since the soap cloud produced by the very small quantity of calcium available in, say, 5 cc. of blood is too faint to be read on the colorimeter, the nephelometer is used instead, and, second, that a solution of ammonium stearate is substituted for the castor oil soap. The method, as developed, is short, taking 2 hours for a set of four determinations as against 3 full days by the ashing and gravimetric procedure; it requires but 5 cc. of blood instead of more than 300 cc.; only two instruments are used, the nephelometer and the centrifuge; the reagents employed are easily obtainable, an important factor at the present time; and finally, the results, as will be shown, are accurate to within less than 1 per cent.

[1] Lyman, H., *J. Biol. Chem.*, 1915, xxi, 551.

PROCEDURE.

For Blood.—To draw the blood, a paraffined pipette attached to a hollow needle by a piece of rubber tubing (according to the method of Folin and Denis[2]) is employed. The potassium oxalate is naturally omitted. Run 5 cc. of blood into a small flask containing 15 cc. of trichloroacetic acid, 6.5 per cent, while agitating the flask. Mix and let stand for a few minutes. Filter through a folded calcium-free filter paper. Pipette 10 cc. of the filtrate into an Erlenmeyer flask of about 50 cc. capacity. Add one drop of methyl orange, 0.1 per cent. Add 2 N ammonium hydrate drop by drop until just yellow. Add nitric acid, 0.05 N, dropwise until pink, and then 1 cc. more. Add 1 cc. of oxalic acid, 4 per cent. Add 1 cc. of sodium acetate, 20 per cent, dropwise. Cool under the water tap until a faint cloud appears Shake 10 minutes or stand over night at room temperature, as convenient. Rinse the stopper with a few drops of ammonium oxalate, 0.5 per cent. Pour into a centrifuge tube and centrifuge. Pipette off supernatant liquor. Rinse the flask with 5 cc. of ammonium oxalate, 0.5 per cent, pour into centrifuge tube, stir, rinsing the rod with 0.5 per cent ammonium oxalate, and again centrifuge. Pipette off supernatant liquor. Dissolve precipitate in 5 cc. of 0.1 N nitric acid by means of stirring, and pour into original flask. Agitate a moment to dissolve any precipitate adhering to the walls. Rinse the rod and centrifuge tub with 5 cc. of water, and pour into the flask.

Into another flask of about 100 cc. capacity, pipette 20 cc. of th standard calcium oxalate solution. Pipette 50 cc. and 25 cc respectively of the ammonium stearate reagent into two clear dry beakers. Pour the standard solution into the 50 cc. of reagent, and the unknown into the 25 cc., and pour back twice. Stopper and let stand 10 minutes. Fill both nephelometer tubes with the standard, set the left side at 32 mm., and take a careful reading to be sure that the two sides of the instrument are balanced. Replace the standard on the left with the unknown and read. Care should be taken before reading to remove with a glass rod any bubbles adhering to the walls of the nephelometer tubes.

[2] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 527.

Calculation.

If the unknown is set at 32 mm. and the standard is read, the reading divided by 4 will equal the number of mg. of calcium in 100 cc. of blood. Note that calcium is calculated as calcium and not as the calcium oxide.

For Milk.—Pipette 10 cc. of the well mixed sample into a volumetric flask, a 200 cc. flask for cow's milk or a 100 cc. flask for human milk. Make up to volume with distilled water and mix thoroughly. The mixture is now of approximately the same per cent calcium content as blood. Pipette 5 cc. of this mixture into 15 cc. of trichloroacetic acid, 6.5 per cent, let stand, filter, and proceed as with blood. To find the number of mg. of calcium per 100 cc. of milk, the nephelometer reading is multiplied by 5 or by 2.5, according as the milk was originally diluted to 200 or 100.

Reagents, Etc.

Trichloroacetic Acid Solution, 6.5 Per Cent.—This reagent, suggested by Greenwald,[3] is used for the coagulation of both blood and milk, three volumes of the reagent being taken for one of the substance to be coagulated. It gives a water clear filtrate, free from protein, and, being highly dissociated, holds in solution any calcium salts present. It, as well as all other materials, should be tested for calcium before use.

Filter Paper.—As the filter paper used for common laboratory purposes contains considerable calcium, a paper must be selected for filtering the coagulated blood or milk which has been washed in acid. For this purpose Baker and Adamson's paper "A" washed in hydrochloric and hydrofluoric acids was employed For filtering the reagents absorbent cotton washed first with hydrochloric acid, 10 per cent, then with water, until the wash water is no longer acid to litmus, and finally dried, may be used.

Indicator, Methyl Orange, 0.1 Per cent.—Dissolve 0.1 gm. of methyl orange in 10 cc. of alcohol and make up to 100 cc. with water.

Nitric Acid, 0.1 N and 0.05 N.—The concentrations need not be exact, provided the same strength of acid is used in all steps

[3] Greenwald, I., *J. Biol. Chem.*, 1915, xxi, 61.

of the process, including the making up of the standard. For convenience a stock solution of 2 N may be prepared and the lower concentrations made up from this as needed.

Ammonium Hydrate, 2 N.—This need not be accurate. 13.5 cc. of ammonium hydrate, sp. gr. 0.9, made up to 100 cc., with water will serve the purpose.

Oxalic Acid, 4 Per Cent.—As 1 cc. of this solution is used to precipitate the calcium in only 2.5 cc. of blood, it will easily be seen that the excess over the theory is very large. Unless such an excess is present, however, precipitation does not begin promptly, owing to the low concentration of calcium oxalate.

Sodium Acetate, 20 Per Cent.—20 gm. of crystallized sodium acetate dissolved in 100 cc. of water.

Ammonium Oxalate, 0.5 Per Cent.

Ammonium Stearate Reagent.—Dissolve 4 gm. of stearic acid and 0.5 cc. of oleic acid in 400 cc. of hot alcohol. Add 20 gm. of ammonium carbonate dissolved in 100 cc. of hot water and allow the mixture to boil for a few moments. Cool. Add 400 cc. of alcohol, 100 cc. of water, and 2 cc. of ammonium hydroxide (sp. gr. 0.9). Filter. This solution should be as clear as freshly distilled water and perfectly colorless. If well stoppered it keeps indefinitely. Before using for analysis, test as follows: Into two flasks pipette respectively 10 and 5 cc. of the calcium oxalate standard and to the 5 cc. add 5 cc. of nitric acid, 0.05 N. Treat both with 25 cc. of the ammonium stearate reagent and read on the nephelometer. If they do not read exactly 2 to 1 there is some impurity present in the chemicals used. The alcohol—if, as is usual in laboratories, it has stood in a wooden barrel—will give a yellow coloration with ammonia and will contain suspended particles which reflect light in the nephelometer. It should be redistilled with a little calcium carbonate. Stearic acid may be purified by recrystallizing from boiling alcohol. Ammonium carbonate may be resublimed.

Calcium Oxalate Standard, 10 Cc. to Contain 0.2 Mg. of Calcium in 0.05 N HNO_3.—Dissolve 72.9 mg. of pure calcium oxalate ($CaC_2O_4 + 1H_2O$) in 25 cc. of nitric acid, 2 N, and make up to 1,000 cc. with water. Since the presence of chlorides affects the solubilities of calcium soaps, nitric acid is used as a solvent throughout instead of hydrochloric.

Accuracy of the Method.

The method was checked as follows: (1) 5 cc. portions of the calcium oxalate standard (equivalent to 0.1 mg. of calcium) were run through the described procedure, including the preliminary treatment with trichloroacetic acid solution. Since half the trichloroacetic mixture is taken, only 0.05 mg. of calcium was being determined at the end. The nephelometer readings against 10 cc. of the standard (0.2 mg. of calcium) were exactly 8:32, giving a calcium content for the solution of 2 mg. per 100 cc., which was correct. No calcium was therefore lost and, since all solutions and materials had been carefully tested for calcium, none had been added. Next, a solution of calcium chloride was made up and a portion analyzed by the gravimetric method. From the data thus obtained the solution was then diluted to contain 5 mg. of calcium per 100 cc., and two 5 cc. portions were run through the method. Respectively 4.96 and 4.94 mg. per 100 cc. were recovered.

(2) Samples of blood taken from the same animal, as nearly as possible at the same time, were ashed and done by the new method. No attempt was made to perform the gravimetric procedure. Such large amounts of blood, at least 300 to 500 cc., must be handled that the loss by spattering and fusing renders this method less accurate than the new one which it is being used to check.

Portions of blood were drawn from the living animal into paraffined pipettes, the first sample being run into a platinum dish for ashing, the second into trichloroacetic acid solution for treatment by the new method, the third into platinum, etc. The samples in platinum were then dried over night on a hot plate and cautiously ashed by hand over a Bunsen burner. The residue was taken up with nitric acid, 0.1 N, and the calcium precipitated as oxalate, washed, treated with stearate solution, and read against the standard on the nephelometer. Much trouble was experienced with this mode of ashing. In the first place the results were not concordant and in the second the values were almost always below (never above) the values obtained on the same blood by the new method. There are three obvious sources of error: (*a*) If an oxidizing mixture, such as

nitric and sulfuric acid, is employed there is invariably a fine spray thrown off at the moment when the ash becomes dry, as may be shown by placing a metal plate about the crucible or dish. This same objection holds when successive small portions of water are used, as is sometimes done. (*b*) If no oxidizing agent is present it is very difficult to burn the residue free from carbon, and if this is not done completely the blood charcoal left has such strong adsorptive power as to make it impossible to extract the calcium salts by simple treatment with dilute mineral acid. (*c*) If much heat is employed the residue fuses in a thin layer over the surface of the dish and can only be removed by mechanical means; *e.g.*, the usual scrubbing with sand. Table I, taken from five consecutive experiments, will serve as an illustration.

TABLE I.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	5.9 5.9	4.6 4.0
" 2	5.83 5.82 5.83	5.78
" 3	5.82 5.81	5.84 4.88
Man	6.12	5.9 5.6
Cat 4	6.16	4.56

At the suggestion of Dr. R. D. Bell, ashing was next attempted by means of the hot air bath alone, without use of the free flame, and this proved perfectly successful. Samples of blood in platinum, taken as before, were first dried in a bomb furnace at about 200°C. for 6 or 7 hours. The temperature was then gradually raised to the full heat of the furnace, 320°C., and maintained there for 4 hours, by which time most of the tarry bodies had been

distilled off. Next the dishes were transferred to a muffle and the muffle was kept at a dull red heat for 4 hours more, the dishes being supported on small tripods to prevent their touching the walls or floor of the muffle. The residue, a reddish powder, was then taken up with nitric acid, 0.1 N, and run through the method as before. Table II gives the result of six consecutive experiments.

TABLE II.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	6.71	6.73
" 2	7.62	7.60
Rabbit	8.37	8.37
Man (1)	6.13	6.13
" (2)	6.06	6.14
" (3)	5.54	5.52

Tests for iron, after the calcium had been thrown down as oxalate and the precipitate washed, were uniformly negative.

One possible source of error then remained to be examined. It is conceivable that the trichloroacetic acid might dissolve some protein-like substance from the blood, which could be carried through the various precipitations and finally thrown down by the large excess of ammonia present in the ammonium stearate reagent. This might offset a possible loss of calcium, although, of course, the probability of the two errors so exactly balancing each other is slight. If, however, such a hypothetical protein body were destroyed by ashing the filtrate from the trichloroacetic acid precipitation, a determination of calcium in this ash would yield lower figures than where the proposed method was carried straight through on another sample of the same blood. That such is not the case is shown in Table III.

(3) The milk determinations were checked up exactly as with blood. After the samples had been diluted as indicated (p. 171), 5 cc. portions were pipetted into platinum dishes for ashing and into trichloroacetic acid solution for determination by the new method. Since, however, the residue from such small amounts

TABLE III.

Calcium Recovered per 100 Cc. of Blood.

Animal No.	New method.	Ashing.
	mg.	*mg.*
Cat 1	7.10	7.12
" 2	5.82	5.86
Rabbit 1	5.84	5.80
" 2	8.43	8.44
Man	6.22	6.20

of milk could be readily burned, the free flame was employed instead of the hot air bath. Table IV gives the result of seven such experiments on milk from different sources. It may be remarked that Sample 2, which shows an unusually low calcium content, came from a somewhat questionable source and possibly had been diluted.

TABLE IV.

Calcium Recovered per 100 Cc. of Milk.

Sample.	New method.	Ashing.
	mg.	*mg.*
1	99.6	99.3
2	86.6	86.6
3	127.5	127.0
4	121.5	121.5
5	122.5	123.5
6	129.5	130.0
7	127.0	127.5

DISCUSSION.

When the work was first begun on this method it was thought that by using a large excess of potassium oxalate it might be possible to precipitate the calcium in both blood and milk directly, thus avoiding the steps of coagulation and filtering. The results were, however, uniformly too low in both cases. In blood either part of the calcium is in chemical combination, perhaps with a protein, or else the blood proteins act as a protective colloid, thus preventing the calcium oxalate from coming down.

This is shown by the following experiment. A wide-mouthed bottle, holding 2,000 cc. and containing 20 gm. of finely powdered potassium oxalate, was filled with ox blood, directly from the vein of the animal, and thoroughly shaken. It was then left in the ice box at 3°C., for 3 days, again shaken, and a portion centrifuged. 50 cc. of the top fluid, which should have been calcium-free, were then run into 6.5 per cent trichloroacetic acid solution, allowed to stand a short time, and filtered. 150 cc. of the filtrate were evaporated to dryness in a large platinum dish and the residue was cautiously burned. The ash was taken up with 0.1 N nitric acid and the calcium in this solution determined by the new method. 0.60 mg. of calcium per 100 cc. of blood was recovered, or a little over 10 per cent of the original calcium content of the blood. The blood of a cat treated in the same manner yielded 1.87 mg. of calcium per 100 cc. of blood, or 31 per cent. A considerable amount of calcium had therefore escaped precipitation as oxalate.

In milk, as has been shown by Van Slyke and Bosworth,[4] the calcium is partly in true solution, partly in a colloidal state, but chiefly combined with protein, probably as calcium caseinate. This being the case, the low results obtained were what might have been expected. Attempts at direct precipitation were therefore abandoned, and, on the suggestion of Professor Folin, the trichloroacetic acid coagulation was adopted instead. Apparently this treatment is sufficient to liberate the calcium from its caseinate in milk and from its protein combination in blood, as well as to hold the inorganic salts in solution. In the precipitation of calcium oxalate from its acid solution McCrudden's[5] technique has been followed, modified of course for the low concentration here met with. This manipulation was based on the principles laid down by Richards[6] and is now too well known to need comment. Fortunately the great insolubility of calcium oxalate in dilute solutions of ammonium oxalate permits very small quantities of this salt to be handled without perceptible loss.

[4] Van Slyke, L. L., and Bosworth, A. W., *J. Biol. Chem.*, 1915, xx, 135.
[5] McCrudden, F. H., *J. Biol. Chem.*, 1911, x, 187.
[6] Richards, T. W., McCaffrey, C. C., and Bisbee, H., *Proc. Am. Acad. Arts and Sc.*, 1901, xxxvi, 377.

The development of the ammonium stearate reagent involved several principles worthy of mention. In the first place, as the calcium soap of castor oil is slightly soluble in the large excess of alkali necessary to hold even the potassium soap in solution, the clouds produced by small amounts of calcium were not quantitative and it became necessary to find a soap of lower solubility. After trial of palmitates, oleates, and some others without success, it was found that calcium stearate would answer the purpose, provided a solution of some salt of stearic acid could be prepared which would remain perfectly clear for a reasonable length of time. Commercially, three agents are commonly employed in the manufacture of liquid soaps, to keep the solution from hydrolyzing: first, excess of alkali, preferably carbonate; second, alcohol; and third, oleates, which have the remarkable power of restraining the hydrolysis of other soaps. The insolubility of ammonium stearate is such that it was found necessary to use all three of these, as may be seen by reference to the description of this reagent.

The large excess of stearate in proportion to the calcium to be precipitated is to be noticed. If this excess is not present, the calcium soap, instead of remaining suspended in an even cloud, settles rapidly to the bottom, either as a flocculent mass, or as a crystalline precipitate, and is of course worthless for nephelometric determination. Finally, the saponification of fats or oils rich in stearates by sodium or potassium hydroxide was found to yield an unsatisfactory reagent, and the method of direct union of stearic acid with ammonia in hot alcohol was selected instead. Aqueous ammonium hydrate is not suitable for this purpose.

In closing I should like to point out that as the introduction of 0.002 mg. of calcium in the course of the determinations would result in an error of 1 per cent, the materials used must be of the highest purity. It is well, occasionally, to run a sample of the standard through the whole procedure to be sure that there is neither gain nor loss at the end.

STUDIES ON ANIMAL DIASTASES.

I. THE INCREASED DIASTATIC ACTIVITY OF THE BLOOD IN DIABETES AND NEPHRITIS.

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Although it has been recognized since the time of Magendie[1] that blood possessed active diastatic properties, no studies of a conclusive nature appear to have been published relative to the activity of the blood diastase in human subjects suffering from diabetes. From an investigation of this question on human diabetics, Foster,[2] Bainbridge and Beddard,[3] Schlesinger,[4] Wynhausen,[5] Ghedini,[6] and Stocks,[7] were unable to come to any conclusions, though Loewi[8] regarded the diastase of considerable importance, and Moeckel and Rost[9] state, apparently from meager data, that the diastatic activity of the blood in human diabetes is almost always higher than normal.

The results obtained on depancreatized dogs have likewise been contradictory, although Milne and Peters,[10] in a comparatively large series of experiments carried out on the dog, uniformly obtained a decided increase in the diastase of the blood after removal of the pancreas. We have obtained similar results on one of Dr. Murlin's depancreatized dogs.[11]

Milne and Peters state: "The comparison of these results with what is found in diabetes is of interest, as it might well be that the excessive amount

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[1] Magendie, *Compt. rend. Acad.*, 1846, xxiii, 189.

[2] Foster, M., *J. Anat. and Physiol.*, 1867, i, 107.

[3] Bainbridge, F. A., and Beddard, A. P., *Biochem. J.*, 1907, ii, 89.

[4] Schlesinger, W., *Deutsch. med. Woch.*, 1908, xxxiv, 593.

[5] Wynhausen, O. J., *Berl. klin. Woch.*, 1910, xlvii, 1281.

[6] Ghedini, G., *Clin. med. ital.*, 1912, li, 146.

[7] Stocks, P., *Quart. J. Med.*, 1916, ix, 216.

[8] Loewi, O., *Marburger Sitzber.* (Nov.), through *Biochem. Zentr.*, 1905-06, iv, 271.

[9] Moeckel, K., and Rost, F., *Z. physiol. Chem.*, 1910, lxvii, 433.

[10] Milne, L. S., and Peters, H. LeB., *J. Med. Research*, 1912, xxvi, 415.

[11] Our thanks are due to Dr. Murlin for placing this material at our disposal.

of sugar in the blood is produced by an increased diastatic action of the serum causing an excessive conversion of glycogen into glucose." They do not appear, however, to have studied this question on cases of human diabetes. It is also worthy of note in this connection that Lesser[12] found that the postmortem glycogenolysis of the liver of depancreatized frogs was three times the normal.

It has been recognized for some time that nephritis may be accompanied by a decrease in the excretion of diastase in the urine, which fact has, indeed, been utilized as a means of determining the impairment in renal function.[13] That this decreased excretion of the diastatic enzyme is accompanied by an accumulation in the blood has been shown by Lœper and Ficaï,[14] Hirata,[15] and Stocks,[7] the last of whom has reported a number of observations with the Wohlgemuth method on cases of nephritis in human subjects.

The paucity of reliable data on the subject of the diastatic activity of the blood appears to be due to the lack of a sufficiently delicate and accurate method. Of the different methods employed, those dependent upon the estimation of the reducing sugars formed are the most satisfactory. The procedure introduced by Lewis and Benedict,[16] for the estimation of the sugar of the blood has been found useful in the estimation of the diastatic activity.

Method.

Two 2 cc. samples of oxalated blood are taken, one being employed as a control. The control tube[17] is made up to 10 cc. with distilled water, and the tube to be employed for the test to 9 cc. Both tubes (cylindrical centrifuge tubes) are now placed in a water bath at 40°C. As soon as the contents of the tubes have been brought to this temperature, 1 cc. of 1 per cent soluble starch is added to the second tube, the contents are mixed, and incubation is then carried out for exactly 15 minutes at 40°C. After

[12] Lesser, E. J., *Biochem. Z.*, 1913, lv, 355.
[13] See Geyelin, H. R., *Arch. Int. Med.*, 1914, xiii, 96.
[14] Lœper, M., and Ficaï, J., *Arch. méd. exp.*, 1907, xix, 722.
[15] Hirata, G., *Biochem. Z.*, 1910, xxviii, 23.
[16] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.
[17] If a sugar estimation is being made simultaneously, there would appear to be no good reason for running a control, since a 15 minutes' incubation results in no appreciable change in the blood sugar. The control may, however, be made the basis of the blood sugar estimation.

the incubation has been completed, about 1 gm. of dry picric acid is at once added to each tube and the mixtures are stirred. When the proteins are precipitated, the tubes are centrifuged and the yellow supernatant fluid is filtered. The sugar in 3 cc. portions of the filtrates is now estimated according to the technique described by Myers and Bailey.[18] Correction is made for the sugar originally present in the blood (with the aid of the control) and for the slight reducing action of the soluble starch. The results have been recorded in terms of the percentage of the soluble starch (10 mg.) transformed to reducing sugars (calculated as glucose) by the 2 cc. of blood employed.

TABLE I.

The Influence of Varying the Amount of Substrate on the Diastatic Activity of 2 Cc. of Blood.

Case.	Blood sugar.	Amount of soluble starch employed.			
		10 mg.	20 mg.	30 mg.	40 mg.
		Reducing sugar formed.			
	per cent	*mg.*	*mg.*	*mg.*	*mg.*
N. L.	0.50	4.65	4.66	4.63	4.46
L. O.	0.46	4.57	4.36	4.75	4.54
G. B.	0.28	4.47	4.36	4.45	4.44
F. P.	0.28	4.28	4.57	4.27	
R. T.	0.11	2.27	2.26	2.37	2.26
		2.30*	2.25*	2.30*	2.25*

* Glycogen employed.

It is believed that 10 mg. of starch furnish a sufficient substrate for all ordinary conditions with human blood (except possibly in very severe diabetes), since practically identical results have been obtained with amounts of starch up to 40 mg., as shown in Table I. Glycogen offers no advantage over soluble starch, since the results are essentially identical. This is evident from the data given in Table II. The possible error of glycolysis would appear to be an entirely negligible one during a 15 minute period of incubation.

[18] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

The scheme we have followed in recording our results is an arbitrary one, but the figures obtained, ranging from 15 to 75, are satisfactory for expressing the variations in the diastatic activity of human blood. If it is desired to express the data in mg. of reducing sugar formed per 100 cc. of blood, this may readily be done by multiplying the above figures by 5. Some such method of recording the results would, obviously, be necessary in comparing the activity of the blood with that of other body tissues. It is believed that the salt content already present in the blood is sufficient to exercise a satisfactory activating influence on the diastatic enzyme.[19]

TABLE II.

The Comparative Diastatic Activity on Soluble Starch and Glycogen.

Case.	Blood sugar.	Soluble starch 10 mg.	Glycogen 10 mg.
		Reducing sugar formed	
	per cent	*mg.*	*mg.*
F. P.	0.55	7.39	7.40
H. I.	0.17	4.23	4.12
F. M.	0.16	4.35	4.40
W. H.	0.16	3.37	3.24
A. K.	0.16	1.73	1.72
R. T.	0.11	2.27	2.30

DISCUSSION OF DATA.

The data which have been collected on the diastatic activity of human blood[20] have been arranged in tabular form under four general headings: normals, diabetics, nephritics, and miscellane-

[19] For discussion of factors which need to be borne in mind in estimating diastatic power, see Sherman, H. C., Kendall, E. C., and Clark, E. D., *J. Am. Chem. Soc.*, 1910, xxxii, 1073, and Kendall, E. C., and Sherman, H. C., *ibid.*, 1910, xxxii, 1087.

[20] We are indebted to Dr. Edward Quintard, Director, and to Drs. A. F. Chace and Ludwig Kast, and other members of the staff of the Department of Medicine for placing these cases at our disposal, and to Dr. R. C. Hood of the Laboratory Interne Staff, for valuable assistance. Our thanks are likewise due to members of the *Laboratory* Interne Staff for serving as our controls.

ous conditions. The results of other blood analyses, *viz.*, the sugar and CO_2-combining power and the non-protein nitrogenous constituents, have likewise been included for the reason that they furnish comparative data, and further, constitute an excellent index of the condition of the different cases.

The findings on six normal men are given in Table III. It will be noted that the activities in these subjects fall within narrow limits, 15 to 17. From the data that we have obtained, it would seem reasonable to conclude that normally the diastatic activity of the blood should not rise above 25, and an even safer limit might be between 15 and 20. With regard to the other analytical data, it will be noted that the uric acid for the last case appears

TABLE III.

The Diastatic Activity of the Blood in Normal Subjects.

Subject.	Age	Sex	Diastatic activity.	Blood sugar	Uric acid	Urea N	Creatinine.
					Per 100 cc		
	yrs.			*per cent*	*mg.*	*mg.*	*mg.*
C. E. W.	25	♂	17	0 12	3 9	13 2	1.4
W. O. H.	24	♂	17	0 11	3 0	13 6	2.3
J. A. K.	25	♂	17	0 10	3 3	14.8	1.1
A. L. L.	27	♂	17	0 10	4 3	14.1	1.1
R. C. H.	28	♂	16	0 10	3 3	14 1	1.2
W. M. H.	29	♂	15	0 11	4 8	12 8	1.0

high, and it may be said, concerning this subject, that he had recently recovered from a mild attack of acute nephritis.

By reason of the marked contrast to the normal and their importance, the observations on the diastatic activity of the blood in diabetes will next be considered. Data on thirteen cases have been collected in Table IV. Determinations of the non-protein nitrogenous constituents were made in many of these cases, but as the figures were essentially normal, they have not been included. It will at once be noted that the diastatic activity is uniformly from two to four times the figure normally observed —figures from 39 to 74. It will further be observed that, with the exception of Case 4, there is a rather close relationship between the blood sugar and the diastatic activity. Case 4 was

on a rigidly restricted diet throughout the period of observation and this would serve to explain the comparatively low figures for blood sugar with the high diastase figures, while, on the other hand, the high diastatic activity affords an explanation as to why the restriction in the diet was unable to bring the blood sugar down to normal. It should, perhaps, be noted that on October

TABLE IV.

The Diastatic Activity of the Blood in Diabetes.

Case.	Age.	Sex.	Date.	Diastatic activity.	Blood sugar.	CO_2-combining power (Van Slyke).
	yrs.		*1916–17*		*per cent*	
1. F. P.	51	♂	Nov. 17	74	0.55	54
			" 24	47	0.28	
2. S. I.	52	♂	Dec. 6	64	0.40	
3. L. O.		♀	Nov. 28	59	0.52	58
4. F. M.	26	♂	" 10	59	0.17	49
			" 14	44	0.16	56
			" 17	43	0.17	
			" 21	39	0.18	
			" 24	41	0.20	
5. G. A.	53	♂	" 26	57	0.44	
6. J. B.	65	♂	Dec. 23	56	0.30	
7. R. G.	57	♀	Nov. 1	53	0.43	56
8. I. A.	56	♀	" 1	53	0.26	60
9. F. A.	51	♀	Oct. 28	51	0.34	
			" 10	49	0.22	50
10. N. L.	55	♀	" 27	47	0.50	
			Dec. 6	46	0.28	
11. F. B.	47	♂	Nov. 29	45	0.28	56
12. T. H.	71	♂	Jan. 3	42	0.23	
13. L. M.	62	♀	" 3	42	0.26	
			" 9	24	0.12	

22 the blood sugar in this case was 0.29 per cent. A diastase estimation was, unfortunately, not made at that time. Case 13 cleared up quickly after admission to the hospital, and it will be observed that the fall in the diastatic activity corresponds with the fall in blood sugar. Higher blood sugars in one case than another, with the same diastatic activity, would appear to depend

largely upon the supply of carbohydrate (glycogen) available. The figures for the CO_2-combining power (Van Slyke), no one of which is much below normal, are apparently without special significance in this series.

A recent observation bearing on the influence of the administration of alkali may be of interest in this connection. The diastatic activity of the blood in a severe case of diabetes (J. S.), who was taking large quantities of sodium bicarbonate, was 26 and the blood sugar 0.44 per cent. Upon discontinuing the alkali therapy for 40 hours the blood diastase rose to 71 and the blood sugar to 0.58 per cent. The patient was again put upon alkali, but died several days later, before we were able to secure another specimen of blood. Although the question of the action of the alkali is important, the data we have so far been able to collect do not warrant a discussion of this subject. We have, however, made a few observations upon the action of alkali, and also of ether anesthesia, upon the blood diastase and sugar.

The diastatic activity of the blood has also been determined in two cases of carcinoma of the pancreas. In one (T. L.) a blood diastase of 45 and a blood sugar of 0.15 per cent was found, and in the other (D. M.) a diastase of 45 and a blood sugar of 0.14 per cent.

That diabetes is not the only condition in which an increase in the diastatic activity of the blood may be found, is shown by the data on cases of nephritis given in Table V, more than half of which gave abnormally high activities. In Case 1 the figure was 52 just before death. It will be noted that the cases with the increased diastatic activity almost invariably showed a hyperglycemia, and it would seem that the diastase stood in a causal relation to this. Attention was called, a year ago, to the high blood sugars frequently encountered in nephritis,[18] but at that time we were unable to offer a satisfactory explanation for the hyperglycemia. Judging from a comparison of the data on the diastatic activity and the urea, it would appear that nitrogen retention was generally accompanied by an increase in the blood diastase, probably due, likewise, to an inefficiency on the part of the kidney. In Cases 1, 3, and 11, in which a series of analyses was made, it will be noted that there is a striking parallelism

TABLE V.

The Diastatic Activity of the Blood in Nephritis.

Case.	Age.	Sex.	Diastatic activity.	Blood sugar.	Uric acid.	Urea N.	Creat-inine.
					Per 100 cc.		
	yrs.			*per cent*	*mg.*	*mg.*	*mg.*
1. M. P.	25	♂	38	0.17	8.0	85	8.1
			41	0.15		123	11.6
			44	0.27		141	14.4
			52	0.22		156	12.1
2. E. P.	34	♀	42	0.21	11.0	85	17.6
3. J. B.	34	♂	32	0.14		60	7.5
			32	0.14	4.4	62	7.3
			41	0.16	6.0	135	9.7
			38	0.16	6.3	110	12.5
4. W. McD.	64	♂	35	0.16	6.3	30	2.7
5. M. B.	60	♀	34	0.16		85	3.2
6. C. K.	20	♀	34	0.18	2.3	16	2.1
7. D. S.	64	♂	33	0.20		86	4.1
8. J. G.	30	♀	31	0.13		22	5.0
9. N. W.	17	♀	30	0.32		72	6.1
10. J. v. M.	72	♂	30	0.14	4.5	20	1.8
11. J. M.	41	♂	21	0.13		28	2.9
			22	0.13	7.3	28	3.5
			25	0.11		39	1.9
			30	0.16	6.4	47	3.6
12. F. K.	42	♀	29	0.18	2.3	16	2.1
13. A. N.	54	♂	28	0.14		32	2.3
14. T. N.	43	♂	27	0.13	5.5	23	2.2
15. K. W.	42	♀	27	0.12	4.4	14	1.9
16. A. P.	40	♂	26	0.12		35	3.4
17. R. M.	29	♀	24	0.12		16	2.0
18. F. K.	43	♀	23	0.14		20	2.8
19. R. A.	30	♀	22	0.13	8.3	68	18.7
20. B. B.	23	♂	22	0.12		17	1.9
21. M. Y.	64	♂	21	0.14	5.5	29	1.5
22. J. G.	55	♂	20	0.14		16	2.5
			17	0.12	8.1	20	2.3
23. G. B.	38	♀	13	0.13	7.5	28	1.6

Case 1, CO_2-combining power = (2) 31; (3) 56, after alkali; (4) 44; died.
" 2, " " = 32, died.
" 3, " " = (2) 32.
" 9, " " = 20.
" 11, " " = (3) 47.
" 19, analyses after decapsulation of kidneys.

between the urea and the diastase. Case 9, with the highest blood sugar, showed the lowest CO_2-combining power.

Of the thirty-four cases included in Table VI under miscellaneous conditions, the first eight may be regarded as showing an increased diastatic activity. With the exception of Case 7, they all exhibited a slight hyperglycemia. It is interesting to note that when inquiry was made of Cases 3 and 7, both of whom were physicians and whose blood had been examined for reasons other than the blood sugar, a history of a previous transient mild glycosuria was obtained. Cases 1, 2, and 4 show a decided hyperglycemia, but the meager histories on these cases give no evidence of a glycosuria. Of the remaining twenty-five cases with diastase figures below 25, only three, Cases 16, 24, and 32, exhibit a decided hyperglycemia.

It would hardly seem profitable, in the present communication, to enter into an extended discussion of the rôle of diastases in metabolism. It should, perhaps, be noted here that the term diastase has been used in preference to amylase or glycogenase, for the reason that diastase is a broader term and might be regarded as covering action on both starch and glycogen.

Some text-books in mentioning the diastase of the blood state that the amount present is too small to be of any practical importance. It may be said, in this regard, that the 6 liters of blood of a normal subject of 70 kilos could convert 1 kilo of starch to sugar in 24 hours, if the activity of the blood remained at 20, according to the above method, during that time.

The character of the blood sugar curve, after the administration of glucose, has been the subject of a number of investigations. The blood diastase may possibly be the important determining factor here. The increased diastatic activity may likewise explain the inability to secure a storage of glycogen in the liver of diabetic animals.[21] It would seem logical to expect that an alimentary glycosuria should occur in individuals with an increased diastatic activity. Furthermore, the diastatic activity of the blood may, indeed, furnish a method of detecting cases of incipient diabetes.

Whether or not the diastatic activity of the blood is normally regulated by some internal secretion, derived, possibly, from the

[21] See Kramer, B., and Marker, J., *J. Biol. Chem.*, 1916, xxiv, p. xxiv.

TABLE VI.

The Diastatic Activity of the Blood in Miscellaneous Conditions.

Case.	Age.	Sex.	Diagnosis and remarks.	Diastatic activity.	Blood sugar.	Uric acid.	Urea N.	Creatinine.
						Per 100 cc.		
	yrs.				*per cent*	*mg.*	*mg.*	*mg.*
1. S. S.	32	♂	Bronchitis, died.	39	0.19		16	2.0
2. W. H.	47	♂	Lues.	34	0.16		13	2.3
3. E. H.	36	♂	Slight albuminuria and cylinduria, glycosuria noted on one occasion.	34	0.16	6.1	13	1.3
				29	0.11	5.1	12	1.7
4. A. M.	42	♀	Syphilis.	30	0.16		18	1.3
5. M. J.	43	♀	Fibrillation.	30	0.14		12	1.1
6. D. D.	38	♂	Gastric ulcer and gall-stones.	29	0.14		15	2.1
7. Z. D.	53	♂	Transient glycosuria noted on two occasions.	29	0.12	6.6	13	1.8
8. A. D.	35	♀	Visceroptosis.	28	0.14		19	1.5
9. O. E.	27	♂	Right side hemiplegia.	25	0.12		25	2.4
10. I. O'L.	36	♂	Sciatica.	24	0.12		20	2.0
11. S. S.	28	♂	Incipient tuberculosis.	23	0.13		14	1.7
12. R. T.	29	♂		23	0.11	5.8	14	1.3
13. J. R.	37	♂		23	0.13	6.2	20	1.2
14. D. A.	18	♂	Syphilis.	23	0.12	4.3	14	1.8
15. J. S.	53	♂	Carcinoma of stomach.	21	0.13		21	1.5
16. E. S.	45	♀	Infectious arthritis.	21	0.17		15	2.1
17. L. H.	32	♂	Tabes.	21	0.12		21	2.1
18. P. S.	50	♂	Duodenal ulcer.	21	0.12		19	1.4
19. R. M.	39	♀	Enteroptosis.	20	0.10		13	1.5
20. M. O.	27	♂	Chronic constipation.	20	0.12	5.0	17	2.6
21. B. P.	18	♀	Cystic ovaritis.	19	0.12		11	1.9
22. S. L.	37	♂	Neurasthenia.	18	0.12	3.2	12	1.4
23. M. S.	35	♂	Duodenal ulcer.	17	0.12		22	2.0
24. E. J.	49	♂	Cardiac with pleural effusion.	17	0.16		27	1.8
25. V. R.	37	♀	Lues, enteroptosis, and pyloric stenosis.	17	0.12		22	1.9
26. H. L.	64	♂	Hypertrophied prostate, pneumonia, died.	16	0.14	5.8	21	3.5
27. M. H.	57	♀	Raynaud's disease.	16	0.13	1.4	14	1.3
28. L. B.	55	♀	Lues.	16	0.11		11	1.1
29. D. S.	47	♂	Hypertension.	16	0.12	6.1	20	1.7
30. M. M.	55	♂	Hypertrophied prostate.	15	0.12	4.5	13	2.4
31. A. K.	28	♂	Pericarditis and arthritis.	15	0.13		17	2.3
32. B. C.	35	♀	Luetic cirrhosis.	15	0.16		12	1.6
33. H. D.	72	♂	Hypertrophied prostate, carcinoma of sigmoid.	15	0.12	6.1	22	1.9
34. J. B.	24	♀	Enteroptosis.	14	0.14		18	1.4

pancreas, it is impossible to say. For a discussion of the internal secretions in this connection, reference may be made to Macleod.[22]

Our studies on diastatic activity with this method were originally begun upon the ptyalin of the saliva, and it is planned to present a report of this work shortly. Other studies are in progress.

SUMMARY.

A simple method for the determination of the diastatic activity of human blood is described.

The diastatic activity of the blood in six normal human subjects ranged from 15 to 17 with this method. From the study of a considerable number of miscellaneous hospital cases, it would appear that the upper normal limit was 25. In a series of 13 cases of diabetes, the figures for the diastatic activity of the blood varied from 39 to 74. In a series of 23 cases of nephritis, 11 showed diastase figures ranging from 30 to 52. Of the 11 cases, 7 exhibited marked nitrogen retention.

The results suggest that the increased diastatic activity in both diabetes and nephritis (as shown by the analyses of the blood) may be the important factor in the production of the hyperglycemia in these conditions.

The increased diastatic activity of the blood in nephritis finds probable explanation in the decreased excretion of diastase in the urine, now well known in this condition, but it is not possible, at present, to offer a satisfactory explanation of the increased diastatic activity of the blood in diabetes.

A fall in the blood diastase would appear to afford a more reliable guide to the efficacy of the dietetic treatment in diabetes than either the blood sugar or urine sugar. Furthermore, an increase in blood diastase may constitute a very early sign of impending diabetes.

[22] Macleod, J. J. R., Diabetes: Its Pathological Physiology, London, 1913, 89.

THE AMINO-ACID NITROGEN CONTENT OF THE BLOOD OF VARIOUS SPECIES.

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The data thus far available upon the quantitative occurrence of amino-acid nitrogen in the blood of different species are far from complete. Furthermore, many of the figures heretofore reported have been obtained after preliminary removal of the blood proteins with alcohol, a procedure which has been shown to be undesirable from the standpoint of accuracy.[1] It was, therefore, believed that it would be of interest to make a fairly complete survey of the question of the quantity of amino-acid nitrogen in various bloods, making use of one method throughout, which had been found to be the most accurate available. The results thus obtained form the subject of the present paper.

Blood from various animals, as well as normal and pathological human blood, was obtained and the amino-acid nitrogen determined by the following method.[2]

Introduce into a flask approximately 0.4 gm. of ground (20 mesh) soy bean, 3 to 5 cc. of water, and 1 cc. of 3 per cent solution of crystallized NaH_2PO_4. Let stand a few minutes with occasional shaking. Run in 20 to 50 cc. of blood and allow to stand at room temperature for 30 minutes. Heat 0.01 N acetic acid to boiling, using five volumes of acid for one of blood. Run in the blood slowly and boil, stirring for ½ minute. Add the same amount of boiling water and boil with stirring for 1 minute. Filter hot through a folded filter and wash the casserole three times with 30 cc. portions of water, heating the water in the casserole and using a rubber-tipped stirring rod. Boil down the filtrate rapidly over a free flame in a casserole. Transfer to a small graduated flask or cylinder, choosing the size so as to obtain nearly the original volume of the blood. Wash the casserole three times with the smallest possible amount of water. The

[1] Bock, J. C., *J. Biol. Chem.*, 1916–17, xxviii, 357.

[2] This method has been previously described in detail by the writer.[1] It is repeated here for the sake of completeness.

volume after washing should not be more than about three-fourths of the final volume. Add enough trichloroacetic acid to make an approximately 3 per cent solution (either the solid acid or a corresponding amount of 50 per cent solution is used). Dilute to volume, shake, and let stand 30 minutes. Add 2 gm. of kaolin, shake well, centrifuge, and filter. Transfer an aliquot part (15 to 40 cc.) of the filtrate to a small flask (50 to 100 cc.), add two or three beads, one drop of alizarin indicator, bring to a boil, and keep slowly boiling (simmering) until the indicator turns. Add 1 to 2 cc. of N potassium hydroxide, and boil 1 to 2 minutes to remove the ammonia. Make acid with a few drops of acetic acid and evaporate to a small volume. The whole amount may be used in the micro Van Slyke apparatus or the liquid transferred to a small graduated test-tube, diluted to a definite volume, and aliquot portions measured out by means of the burette of the Van Slyke apparatus.[3]

The heat coagulation gives clear and easily filterable precipitates with freshly drawn blood; *i.e.*, blood not over 24 hours old. If the blood has been drawn for more than that time, it often happens that the filtrate from this precipitation is not entirely clear and the solution filters very slowly. The same thing has been found with laked or frozen blood. In such cases the blood must be precipitated with nine volumes of 2.5 per cent trichloroacetic acid and treated as described in a previous paper.[1]

The blood of larger animals, such as the ox, calf, sheep, and pig, was obtained from slaughter houses and immediately (1 to 2 hours after slaughtering) used for analysis. The blood of cats and dogs was obtained from the carotid artery. The blood was drawn about 12 hours after the last feeding. Normal human blood was drawn from a vein in the forearm (usually the median basilic).[4] The normal and most of the pathological bloods were drawn 3 to 4 hours after breakfast. The blood of birds was obtained by cutting the throat and letting the blood run into a bottle containing potassium oxalate. 40 to 50 cc. of animal and normal human blood were used for the analysis. The amounts of pathological bloods obtained from the wards varied greatly, but with few exceptions there were always more than 15 cc. used for analysis. These bloods were delivered in small bottles and in order to prevent waste, the blood was weighed out instead of measured, pouring it into the previously tared flask containing the soy bean mixture. The weight in gm. was divided by 1.06, the mean specific gravity of human blood. For the sake of comparison the results are tabulated at the end of the paper.

Table I shows the amino-acid nitrogen content of the blood of various mammals, including the ox, sheep, pig, cat, and dog.

[3] Van Slyke, D. D., *J. Biol. Chem.*, 1913–14, xvi, 121; 1915, xxiii, 407.

[4] The pathological blood was furnished through the courtesy of Dr. A. O. Gettler and other members of the staff of Bellevue Hospital, to whom the writer expresses his thanks.

The figures for each species are constant. Van Slyke and Meyer,[5] using the alcohol precipitation method, find from 3 to 5 mg. of amino-acid nitrogen per 100 cc. of blood of dogs which had been fasting from 20 to 24 hours. Costantino[6] reports findings of 10 mg. of amino-acid nitrogen in 100 gm. of blood obtained from dogs during full digestion. His analysis of pig blood shows 10 mg. of amino-acid nitrogen per 100 gm. of blood. Costantino uses the Sörensen formol titration method after drying the blood at 70°C. and extracting with 10 per cent alcohol in the presence of barium salts. György and Zunz,[7] using the alcohol precipitation method (removing urea by treatment with soy bean and subsequent aeration) find 4.8 mg. of amino-acid nitrogen in 100 cc. of blood obtained from dogs fasted for 24 hours. In the present work, dog blood was found to contain an average of 7.47 mg. of amino-acid nitrogen per 100 cc. of blood, and pig blood 8.43 mg. per 100 cc. of blood. These higher figures in the dog are probably due to the more complete extraction of the amino-acid nitrogen obtained in the present work. We cannot explain Costantino's high results for pig blood.

The amino-acid nitrogen of bird blood (Table II) is, roughly speaking, three times as high as that of mammals. The individual and gross variations are in proportion to those of mammalian blood. Costantino, using the formol method[6] finds 20 mg. of amino-acid nitrogen per 100 gm. of turkey blood, agreeing closely with the findings in the present paper.

The distribution of the amino-acid nitrogen between plasma and corpuscles in certain species was studied by Costantino[6] and by György and Zunz.[7] Costantino finds that serum and corpuscles during fasting are constant in their amino-acid nitrogen. His findings show 4.4 mg. of amino-acid nitrogen per 100 gm. of dog serum, and 100 gm. of the corpuscles of ox blood containing 3.2 mg. of amino-acid nitrogen. Turkey blood analyzed by Costantino showed 3 mg. of amino-acid nitrogen in 100 gm. of serum and 34 mg. in 100 gm. of corpuscles. György and Zunz find 1.7 mg. of amino-acid nitrogen in the plasma of 100 cc. of

[5] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

[6] Costantino, A., *Biochem. Z.*, 1913, li, 91; 1913, lv, 403.

[7] György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.

dog blood and 3.1 mg. in the corpuscles of 100 cc. of the same blood, the corpuscle content being calculated by difference.

In the present work the whole blood was first analyzed. Samples of equal volumes were centrifuged for $\frac{1}{2}$ hour at high speed. The plasma which had separated out was withdrawn by means of a pipette, physiological salt solution added in its place, and the sample was mixed and centrifuged again. This procedure was repeated four times. After all the clear supernatant fluid had been withdrawn down to the light colored layer of leukocytes, the corpuscles were laked with water, transferred to a flask, and the liquid was diluted approximately to the same volume as the plasma plus the washings. As the laked blood gives trouble with the heat coagulation procedure the direct trichloroacetic acid precipitation was used after treatment with soy bean. Table III shows the results obtained from mammalian and bird blood. In mammalian blood the corpuscles show slightly higher figures than the plasma. The difference in bird blood is pronounced, the corpuscles containing about two-thirds of the total amount of amino-acid nitrogen. These findings agree in general with those of the authors mentioned above.

In view of the fact that the corpuscles contain a large proportion of the amino-acid nitrogen of the blood, they should be included in experimental or clinical work on the nitrogen content of the blood.

The normal human bloods are reported in Table IV. It will be noted that the figures are remarkably constant for different individuals. The average figure is 7.13 mg. with a maximum of 7.9 mg. and a minimum of 6.13 mg. per 100 cc. of blood, approximately those of other mammals. Sex appears to be without influence. The placental blood tends to be distinctly higher.

The pathological bloods (Table V) were taken at random from ward cases in Bellevue Hospital and thus serve to show the possible variations found in a wide variety of conditions. In these cases variations occur from 4.5 mg. to 30 mg. per 100 cc. of blood. The most pronounced variations from the normal were found in nephritis. Of three typhoid cases, two are decidedly below normal. Jaundice, cardiac cases, carbuncles, rheumatic fever, hyperthyroidism, and cirrhosis of the liver show an increase over

the normal. The remaining cases investigated give the same results as normal bloods.

The nephritic bloods are being studied further to find whether the amino-acid nitrogen tends to parallel the total non-protein nitrogen in the blood of these cases.

TABLE I.

Amino-Acid Nitrogen per 100 Cc. of Blood.

Sample No.	Source.	N*	Sample No.	Source.	N*
		mg.			*mg.*
4	Ox (oxalated).	6.17	42	Pig (defibrinated).	8.37
125	" "	6.22	43	" "	8.49
10	" "	6.43	Average		8.43
5	" "	6.66			
125a	" (defibrinated).	6.67	14d	Cat "	8.12
9	" (oxalated).	6.89	14b	" "	8.13
3	" "	7.04	14c	" "	8.64
Average.		6.58	14a	" "	9.83
			Average.		8.68
100	Calf (defibrinated).	6.29			
8	" (oxalated).	6.66	97	Dog "	6.68
18	" (defibrinated).	6.81	52	" "	6.87
18a	" "	7.60	113	" "	6.87
Average.		6.84	51	" "	7.15
			114	" "	7.47
6	Sheep (oxalated).	6.84	116	" "	7.88
13	" defibrinated).	7.50	98	" "	8.37
12	" "	7.79	99	" "	8.48
11	" "	7.82	Average.		7.47
7	" (oxalated).	8.19			
Average.		7.63			

* The data are arranged in sequence for each group.

TABLE II

Amino-Acid Nitrogen per 100 Cc. of Blood (Oxalated).

Sample No.	Source.	N*
		mg.
26	Chicken.	17.81
36	"	19.84
23	"	20.64
24	"	21.32
25	"	21.58
110	"	21.93
107	"	23.78
Average...............		**20.99**
35	Duck.	20.22
32	"	20.88
34	"	20.95
33	"	21.55
27	"	22.98
Average...............		**21.32**
101	Turkey.	20.00
102	Goose.	16.97
104	"	17.16
105	"	18.49
103	"	19.20
106	"	21.18
Average...............		**18.60**

* The data are arranged in sequence for each group.

TABLE III.

Sample No.	Source.	Amino-acid nitrogen per 100 cc. of blood.		
		Whole blood.	Plasma.	Corpuscles.
		mg.	mg.	mg.
100	Calf.	5.69	{3.06 3.13	3.59 3.48
100a	"	6.30	{3.33 3.33	3.84 3.28
106	Goose.	21.18	{6.60 6.07	14.78 15.95
107	Chicken.	23.78	{6.63 7.97	14.20 16.00
110	"	21.93	5.31	19.12
125	Ox.	{6.22 6.77	3.05 2.99	4.70 4.87

TABLE IV.

Amino-Acid Nitrogen per 100 Cc. of Human Blood.

Sample No.	Sex.	N*
	Normal venous blood.	
		mg.
47	♂	6.13
15	♂	6.40
57	♂	6.58
60	♂	6.75
58	♂	6.78
77	♂	7.00
83	♂	7.01
86	♂	7.11
21	♂	7.18
30	♂	7.27
55	♀	7.29
48	♂	7.31
16	♂	7.38
22	♂	7.38
19	♂	7.58
65	♂	7.66
56	♀	7.67
46	♀	7.90
Average...............		7.13
	Placental blood.	
20		6.78
38		7.51
28		8.90
31		9.76
138		11.80
139		12.15
Average...............		9.48

* The data are arranged in sequence for each group.

TABLE V.

Amino-Acid Nitrogen per 100 Cc. of Pathological Human Blood.

Sample No	N.	Diagnosis.	Sample No.	N.	Diagnosis.
	mg.			*mg.*	
39	7.78	Syphilis.	85	8.07	Nephritis.
40	7.98	"	87	6.97	Cardionephritis.
44	6.54	Ulcer on penis.	88	8.27	Rheumatic fever.
45	8.34	No symptoms.	89	7.01	Cardiac.
49	8.49	Nephritis.	90	9.38	Nephritis.
50	8.38	"	91	5.72	Carcinoma of liver.
53	7.11	Cardionephritis.	92	7.50	Nephritis.
54	17.50	Nephritis, alcoholism.	93	6.52	"
59	6.77	No record.	94	8.02	"
61	7.27	Nephritis.	95	11.28	Parenchymic nephritis.
62	8.78	No record.	108	8.13	" "
63	8.31	Jaundice.	109	8.04	Nephritis.
64	7.25	Nephritis.	110	8.49	"
66	8.62	Cardiac.	111	7.55	Parenchymic nephritis.
67	7.80	Diabetes.	112	8.69	Cardionephritis.
68	7.86	Cardionephritis.	115	8.87	"
69	6.47	Chronic nephritis.	117	6.66	Nephritis.
70	6.93	Typhoid.	118	8.24	Parenchymic nephritis.
71	8.70	Gout, rheumatism,	120	10.98	Nephritis.
		nephritis.	121	9.82	"
72	5.88	Typhoid.	122	11.17	Colic, uremia.
73	5.45	"	123	8.41	Cardionephritis.
74	4.45	Nephritis.	124	29.98	Uremia.
75	15.10	"	126	9.78	"
76	7.52	Arteriosclerosis.	127	7.25	Nephritis.
78	9.05	Nephritis.	128	7.45	Delirium tremens.
79	5.90	Uremia.	129	9.91	Nephritis.
80	6.05	Nephritis.	130	10.45	Cirrhosis of liver and
81	6.65	"			cardiovalvular case.
82	8.20	Carbuncles.	131	9.58	Hyperthyroidism.
17	6.89	Nephritis.	132	6.46	Parturition.
84	6.24	"	133	6.63	Diabetes.

THE SEPARATION AND ESTIMATION OF BUTYRIC ACID IN BIOLOGICAL PRODUCTS. I.*

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Duclaux[1] has determined approximately quantitatively the individual volatile organic acids of the series from formic to caproic acids, inclusive, by estimating the acidity of the separate distillates. The individual acids are found to distil at a fixed rate, uninfluenced by the presence or absence of other volatile substances. The acids are determined, consequently, by their rate of distillation as a physical constant. This method was used by Jensen[2] to determine the volatile aliphatic acids in cheese, and by Dox and Neidig[3] to determine the volatile aliphatic acids in corn silage. Luck[4] separated formic, acetic, propionic, and butyric acids by the different solubilities of their barium salts in absolute alcohol. Haberland[5] could not duplicate Luck's findings but separated approximately quantitatively acetic and butyric acids by the difference in solubilities in water of their silver salts. The more or less complete solubility of these salts either in the alcohol or in the water renders impossible a quantitative separation of these acids in the manner described in the

* Read at the Fifty-third Meeting of the American Chemical Society in New York, September 25–30, 1916. Published by the permission of the Secretary of Agriculture.

[1] Duclaux, E., *Ann. sc. l'école normale supérieure*, 1865, ii, 270; Traité de microbiologie, Paris, 1900, iii, 384; *Ann. chim. et phys.*, 1874, series 5, ii, 289; 1886, series 6, viii, 542.

[2] Jensen, O., *Landwirtsch. Jahrb. Schw.*, 1904, xviii, 319.

[3] Dox, A. W., and Neidig, R. E., *Iowa Agric. Exp. Station Research Bull.* 7, 1912.

[4] Luck, E.; *Z. anal. Chem.*, 1871, x, 184.

[5] Haberland, K. R., *Z. anal. Chem.*, 1899, xxxviii, 217.

literature. In a previous paper[6] from this laboratory it has been shown that lactic acid may be separated quantitatively from formic, acetic, and citric acids by the fractional distillation of their ethyl esters, and from propionic and butyric acids by the different solubilities of their quinine salts in carbon tetrachloride. The separated lactic acid was estimated as quinine lactate and identified by its melting point. The present paper describes a method for the separation of butyric acid from formic and acetic acids by treating their barium salts with an excess of quinine sulfate, dissolving the quinine butyrate in carbon tetrachloride, *and weighing as quinine butyrate, which is identified by its melting* point.

For the work recorded here the chemically pure carbon tetrachloride of commerce, found free from residue by volatilization of 50 cc., was used. The pure ethyl acetate of commerce was washed with water and dried over calcium chloride before distilling. The portion distilling between 77° and 77.5° was used. Petroleum ether was redistilled and the portion boiling between 55° and 75° reserved for use.

The purest formic and acetic acids of commerce were diluted suitably with distilled water, and the strength of the solution in each case determined by titration with a solution of barium hydroxide of known strength, approximately 0.1 N, with phenolphthalein as an indicator.

Pure butyric acid was prepared from ethyl butyrate, boiling at 120.9–121.1° (corrected) and under 763 mm. pressure obtained by repeated fractionation of commercially pure ethyl butyrate. This was hydrolyzed by heating with an excess of barium hydroxide solution under a return condenser. The excess of barium hydroxide was converted to the carbonate by passing carbon dioxide through the solution, the barium carbonate was removed by filtration, and by boiling the filtrate was freed from carbon dioxide. Dilute sulfuric acid (1:3) was added in excess, the precipitate of barium sulfate was filtered, and the filtrate was distilled under diminished pressure. The distillate containing the butyric acid was diluted to definite volume, and the strength of the solution was determined by titration with a solution of

[6] Phelps, I. K., and Palmer, H. E., *J. Am. Chem. Soc.*, 1917, xxxix, 136.

barium hydroxide of known strength, approximately 0.1 N, with phenolphthalein as an indicator.

Pure propionic acid was prepared from ethyl propionate, boiling at 98.7–99.0° (corrected) at 760 mm. The ethyl propionate was hydrolyzed by heating under a return condenser with an excess of barium hydroxide solution, and a solution of propionic acid was obtained from this in a manner similar to that described above for butyric acid. The strength of the solution of propionic acid was estimated by titration in the presence of phenolphthalein as an indicator, with a solution of barium hydroxide of known strength, approximately 0.1 N.

Pure quinine sulfate and pure quinine were prepared as described in the paper by Phelps and Palmer,[6] to which reference has been made. The quinine salts of formic, acetic, propionic, and butyric acids were prepared by adding the calculated molecular quantities of the pure quinine dissolved in 95 per cent alcohol to the separate acids and evaporating the solution to dryness under diminished pressure at 15 mm. The quinine butyrate was recrystallized from ethyl acetate solution by diluting to twenty times its volume with petroleum ether, when, on standing for a half hour without further stirring, crystals formed. These were gathered on paper and dried in a vacuum desiccator over potassium hydroxide. If the solution was stirred immediately after adding the petroleum ether, the quinine butyrate collected as a sticky mass on the sides of the beaker and was converted to the crystalline form more slowly. The quinine butyrate so obtained had a melting point of 77.5° (uncorrected). The approximate solubilities of the quinine salts of the acids in carbon tetrachloride, together with their melting points, are given in Table I. The solubilities were determined approximately by allowing each substance to stand in contact with a definite volume of carbon tetrachloride in a stoppered flask for 72 hours, filtering, and weighing the residue left on evaporation of the carbon tetrachloride after drying in a water-jacketed vacuum oven at 50°. It is seen from Table I that a separation of quinine butyrate from quinine formate and quinine acetate can be made by the difference in their solubilities in carbon tetrachloride, but the difference in solubility of quinine propionate and quinine butyrate is not sufficient for a complete separation.

TABLE I.

Melting Points and Approximate Solubilities of Quinine Salts.

	Melting point (uncorrected).	Approximate solubility in carbon tetrachloride.
	°C.	
Quinine formate	110.0–113.0	1 in 16,000
" acetate	124.0–126.0	1 " 2,000
" propionate	110.5–111.0	1 " 450
" butyrate	77.5	1 " 25
" sulfate	214.0	1 " 40,000

Definite portions of the solution containing the butyric acid, either alone or in the presence of other acids, were titrated with a solution of barium hydroxide of known strength, approximately 0.1 N, in the presence of phenolphthalein. To the neutral solution was added a slight excess of a solution of neutral quinine sulfate in hot water, the amount of quinine sulfate necessary to precipitate the barium completely as barium sulfate being calculated from the amount of barium hydroxide required. The solution was cooled by standing in ice water and filtered through asbestos as soon as the precipitate of barium sulfate had subsided. The residue was rinsed three times with portions of 10 cc. of distilled water. The filtrate, containing the quinine salts of any of the volatile organic acids that may be present, was evaporated to dryness by distillation under diminished pressure, preferably as low as 15 mm., in order to minimize the formation of quinotoxine from the quinine. The residue was dissolved from the sides of the flask with 10 cc. of alcohol, the alcohol removed by distillation under diminished pressure, and this residue dried by allowing a gentle current of air to pass through the flask. The dry residue, which was generally amorphous, was allowed to stand for 18 hours with 20 cc. of carbon tetrachloride, or with an amount sufficient to dissolve the quinine butyrate which might be present, as shown by the solubility given in Table I. If the original residue was amorphous, supersaturated solutions tended to form and some of the quinine salts of the other acids remained in solution with the quinine butyrate, necessitating retreatment with carbon tetrachloride to separate them. The solution was filtered, and the residue washed on the filter with carbon tetrachloride. The carbon tetrachloride solution, contained in a 100 cc.

Erlenmeyer flask, was evaporated to dryness at room temperature by allowing a rapid current of air to pass over the solution by means of a glass tube suspended just above the surface of the solution. If the residue was amorphous it was dissolved in ethyl acetate and after evaporation at room temperature by a rapid current of air the quinine butyrate was obtained in crystalline condition. To the residue, dried in a vacuum desiccator over potassium hydroxide, 20 cc. of carbon tetrachloride were added, and allowed to stand in the stoppered flask 18 hours to dissolve the quinine butyrate. The solution was filtered and the residue washed with carbon tetrachloride. The undissolved residue was allowed to stand in a stoppered flask for 18 hours with 10 cc. of carbon tetrachloride to dissolve any quinine butyrate which remained, and the solution was then filtered. The filtrates were evaporated at room temperature by allowing a rapid current of air to pass over the solution. When the quinine butyrate obtained in this manner from carbon tetrachloride by evaporation was amorphous, it was dissolved in a small amount of ethyl acetate, from which solution, after evaporation at room temperature by allowing a rapid current of air to pass over the solution, it was obtained in a crystalline form which could be dried in a vacuum desiccator over potassium hydroxide. The dried residue was weighed as quinine butyrate and identified by its melting point. If the melting point showed the presence of impurities, the residue was purified by another treatment with carbon tetrachloride. When it failed to appear crystalline after this purification the quinine butyrate was recrystallized as given above for the purification of the quinine butyrate when prepared for the experimental work. The crystals which formed on standing a few minutes were filtered, dried in the air, and identified by their melting point.

The results given in Table II show that, if the precautions are taken to avoid decomposition of the quinine butyrate by evaporating the solutions at low temperature and drying in a vacuum desiccator over potassium hydroxide, butyric acid may be estimated by weighing as quinine butyrate when present alone or when associated with formic acid and acetic acid, and may be identified as such by its melting point. When propionic acid is present, as in Experiments 11, 12, and 13, it is seen that some of the quinine propionate is dissolved with the quinine butyrate

in the carbon tetrachloride, so that a partial separation only in this way is possible. If the residue from the distillation under diminished pressure was in crystalline form, as was the case in Experiment 3 of Table II, it was found that the tendency of the quinine acetate to form a supersaturated solution in the carbon tetrachloride was so small that a further treatment of the residue from the evaporation of the carbon tetrachloride solution for the separation of these salts was unnecessary. The residue left undissolved by the carbon tetrachloride, however, was allowed to stand 18 hours with another 10 cc. of carbon tetrachloride, in order to dissolve the quinine butyrate completely. This solution was added to the solution obtained by the first extraction, before evaporating to obtain the residue, the purity of which after weighing was found by its melting point.

TABLE II.

Estimation of Butyric Acid after Separation from Formic, Acetic, and Propionic Acids.

No.	Acids taken.				Quinine butyrate found.	Butyric acid calculated.		Melting point of quinine butyrate.
	Formic.	Acetic.	Propionic.	Butyric.				
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*	*°C.*
1				0.1129	0.5271	0.1126	99.7	77.5–78.0
2				0.1382	0.6287	0.1343	97.2	
3		0.0972		0.0691	0.3119	0.0666	96.4	77.5–78.0
4		0.1037		0.1129	0.5310	0.1134	100.5	75.5
5		0.0519		0.1694	0.7395	0.1580	93.2	77.5–78.0
6	0.0804			0.0691	0.2993	0.0639	92.4	77.5–78.0
7	0.1119			0.1129	0.5189	0.1108	98.2	77.0
8	0.0560			0.1694	0.7112	0.1519	89.7	76.5–77.0
9	0.0560	0.0519		0.0565	0.2672	0.0571	101.0	76.0–76.5
10	0.1119	0.1037		0.0565	0.2507	0.0535	94.8	74.5–75.0
11			0.1414	0.1129	0.6042	0.1291	114.3	Softens at 76.0–77.0 Melts at 92.0–93.0
12			0.1414	0.0565	0.3722	0.0795	140.7	92.0–95.0
13			0.0707	0.1694	0.9962	0.2128	125.6	Softens at 75.0–76.5

SUMMARY.

The melting points and approximate solubilities of quinine propionate and quinine butyrate in carbon tetrachloride were determined. The solubilities of the propionate and butyrate

are so nearly the same that only a partial separation can be effected by the method described. The solubility of the propionate is so much greater than that of the formate that propionic and formic acids can be readily separated. It has been shown that butyric acid may be separated from mixtures containing formic, acetic, and butyric acids by means of the solubility of quinine butyrate and the insolubility of quinine formate and quinine acetate in carbon tetrachloride; further, the butyric acid may be estimated by weighing the quinine butyrate, which may be crystallized and identified by its melting point.

THIOBARBITURIC ACID AS A QUALITATIVE REAGENT FOR KETOHEXOSE.

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(Received for publication, January 20, 1917.)

According to Fenton and Gostling,[1] all hexoses are convertible into hydroxymethylfurfural. This substance is, however, much more easily formed from the ketoses than from the aldoses. It is upon this fact that the orcinol, resorcinol, and phloroglucinol tests depend. Fenton and Gostling obtained several times as much bromomethylfurfural from ketohexoses or ketohexose-yielding substances as they did from aldohexoses. By heating with 0.3 per cent oxalic acid under three atmospheres' pressure Van Eckenstein and Blanksma[2] obtained practically a 20 per cent yield of hydroxymethylfurfural from fructose, while from glucose they obtained but 1 per cent. The writer has been able to convert 70 to 75 per cent of fructose into hydroxymethylfurfural by heating with concentrated hydrochloric acid. Under the same conditions sucrose yielded 60 to 70 per cent of the theoretical amount of its fructose in the form of the furfural derivative. The method of determination consisted in precipitating the hydroxymethylfurfural with thiobarbituric acid[3] in the presence of 12 per cent hydrochloric acid, drying, and weighing the precipitate. The product had a reddish orange color and was appreciably soluble in water. Had solubility corrections been applied, the above percentages would no doubt have been higher. Analysis of the product showed a nitrogen content of 11.20 per cent, whereas the

[1] Fenton, H. J. H., and Gostling, M., *J. Chem. Soc.*, 1899, lxxv, 423; 1901, lxxix, 807.

[2] Van Eckenstein, W. A., and Blanksma, J. J., *Ber. chem. Ges.*, 1910, xliii, 2355.

[3] Dox, A. W., and Plaisance, G. P., *J. Am. Chem. Soc.*, 1916, xxxviii, 2156.

calculated value for the condensation product, hydroxymethylfurfuralmalonylthiourea is 11.11 per cent.

When glucose was subjected to the above treatment only a very slight precipitate resulted. Even after long continued heating with acid, 0.2 gm. of glucose gave only 0.001 to 0.002 gm. of precipitate. Lactose and maltose gave somewhat larger amounts of hydroxymethylfurfural, especially after prolonged heating. As suggested by other investigators, this indicates a gradual molecular rearrangement, possibly from aldose to ketose.

On the other hand, prolonged heating of fructose decreases the yield of hydroxymethylfurfural, the latter substance undergoing decomposition into levulinic acid. The yield is likewise slightly decreased by using 12 to 15 per cent hydrochloric acid instead of the concentrated acid. When acid of this strength is used, no precipitate whatever can be obtained from glucose or other aldohexoses.

The thiobarbituric acid test gave positive results when applied to the following carbohydrates, all of which contain a ketose: sucrose, raffinose, tagatose, *l*-sorbose, and inulin. The following gave negative tests: glucose, mannose, galactose, lactose, maltose, starch, cotton, filter paper, glucosamine, α-methylglucoside, arabinose, and xylose.

A noteworthy fact is that the pentoses are not as easily converted into furfural as are the ketohexoses into the corresponding hydroxymethyl derivative. Arabinose and xylose do not form furfural until the acid concentration is almost 20 per cent. However, the presence of furfural can easily be tested in the distillate by means of aniline acetate paper.

It is recommended that the thiobarbituric acid test for ketohexoses be applied as follows: The substance to be tested is placed in a test-tube and sufficient hydrochloric acid and water are added to bring the acid concentration to 12 per cent. The tube is heated over a free flame until boiling begins. It is then cooled under the tap and finally a few drops of a thiobarbituric acid solution (in 12 per cent HCl) are added. If a ketohexose was originally present, an orange-colored precipitate forms on standing. If only aldoses were present, the solution may become yellow but no precipitate results. Barbituric acid cannot be used in place of thiobarbituric, for the reason that the condensation product is much more soluble.

THE GROWTH OF ISOLATED PLANT EMBRYOS.

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Delayed germination or the resting period of seeds presents not only one of the most interesting problems in plant life but also one of the most difficult, owing mainly to the many conditions governing the normal germination of seeds. Space does not permit of even a brief historical sketch of the abundant investigations that have been made concerning this subject. Suffice it to say that these studies have brought forth explanatory theories ranging from zymogenic changes occurring in the embryos and cotyledons to the toughness and non-permeability of the integument to moisture and gases, either or both being more or less dependent on the physical conditions affecting the seed, among which might be named temperature, pressure, moisture, light, and the surrounding media, whether gaseous, liquid, or solid.

From one point of view it would seem that there is a rest period of a longer or shorter duration in the life of most seeds which is limited by internal changes, such as the accumulation of ferments or other chemical changes normally dependent on time, while from another point of view it would seem that there is no real resting period and that germination is delayed because of lack of favorable conditions or proper stimulant. This latter view is more or less proved by the many instances where investigators have greatly shortened the apparent resting period of seeds by physical and chemical methods and have increased the percentage germination of certain seeds by like methods.

Following this line of thought, it might be added that after the rest period is ended the embryo is dependent on the food supply stored in the cotyledons. The young plant consumes part of this food supply which is sufficient to cause it to grow until it

has generated organs capable of utilizing nutriment from external sources—then the cotyledons, being of no further value, shrivel up and finally drop off.

The embryos of different seeds are found to differ widely in size and nature, and the food which serves to nourish them during their early growth varies greatly in character and composition. In the case of the persimmon seed, for example, the embryo consists of two perfectly formed leaves attached to a well defined caulicle—this minute plant draws its early nourishment from cotyledons which, like the seed of the date, closely resemble vegetable ivory in appearance. Among the seeds containing highly specialized embryos might be named the okra, papaw, corn, bean, and many others.

Some years ago during the progress of certain experiments which involved the study of seeds, one of us (Kastle) observed these highly specialized embryos and became interested especially in the embryos of the okra and lima bean. The embryo of the lima bean consists of two perfectly formed leaves attached to a caulicle about 0.75 cm. in length. Finding that this embryo could be easily dissected out, it seemed of general interest to determine if it would grow when deprived of its normal food supply from the cotyledons, and ultimately to establish some of the relations existing between an embryo and the adjoining cotyledons. Other investigations being in progress, it was impossible to continue this research and it was only last spring that during the progress of investigations concerning the study of beans in this laboratory, an embryo of the lima bean was dissected out and planted in moist sand. At the expiration of 24 hours it was noticed that the caulicle had doubled in length but the leaves remained unchanged in size and color while the small bud between the colorless leaves developed a pale green color. There was no further development observed and at the end of 8 days the young plant had dried up and was dead, having developed no lateral roots.

Here was found ample evidence of life and it remained to be determined what substances would nourish the embryo and bring about the normal growth of the plant. With this idea in view a series of experiments was planned to determine the food requirements of isolated lima bean embryos, and to establish the rela-

tions existing between the cotyledons and the embryos during early growth.

Knudson[1] has published some results concerning the toxicity of galactose and the more or less stimulating effect of certain carbohydrates on the vetch, pea, and other seeds. In these experiments he has grown plants from the seeds that had been planted in nutrient solutions containing 1 per cent agar plus different carbohydrates. In his experiments these carbohydrates are supplemental to the food supply stored in the cotyledons, and he does not prove the necessity of any of these compounds for the growth of the young embryo.

In the literature at our command we have failed to find any reference concerning the early food requirements of a seed embryo except that the reserve material stored in the cotyledons, upon hydrolysis, furnishes the nourishment required by the embryo during early growth.

In these experiments perfect beans were selected, care being taken that the integuments were not injured. After carefully removing the integument, the embryo was dissected out under sterile conditions and dropped into sterile distilled water. The embryos were immediately transplanted into test-tubes that were half filled with the different nutrient media, all operations being carried out in a dust-proof closet. The medium in each case was composed of distilled water containing 0.65 per cent of well washed agar plus the different nutrients, as shown in the table.

It will be seen from the table that agar alone does not support the growth of the embryo of the lima bean. The embryo swells to about twice its original size, the leaves turn pale green in 3 days, and then etiolate, and the plant dies in 9 days, no lateral roots having been developed.

When glucose was added to the agar a good growth was obtained, the radicle above the agar and leaves turning green in 48 hours and the growth reaching 2 inches in 14 days, having developed a good root system.

Glucose plus Hopkins' plant food solution gave good growth, the size being greater than when glucose alone was used.

[1] Knudson, L., *Ann. Missouri Bot. Gardens*, 1915, ii, 659.

Lima Bean Embryos.

No.	Media.	Result.
1	Agar (0.65 per cent) in distilled water. Control..................	Very small growth. Dead in 9 days.
2	Glucose (2 per cent)........................	Good growth.
3	" (2 " ") + 1 cc. Hopkins'* plant food solution........................	Best "
4	1 cc. Hopkins' plant food solution............	No "
5	Dry lima bean cotyledons, ground (2 per cent)........................	" "
6	Dry lima bean cotyledons, ground, + 1 cc. Hopkins' solution..................	" "
7	Dry lima bean cotyledons, ground, + 2 per per cent glucose........................	Good "
8	Sucrose (2 per cent)........................	" "
9	Lactose (2 " ")........................	Fair "
10	Raffinose (2 " ")........................	" "
11	Mannite (2 " ")........................	Very small growth. No lateral roots.
12	Maltose (2 " ")........................	Small growth.
13	Starch (2 " ")........................	No "
14	Sprouting green cotyledons (2 per cent) (reducing sugars present)..................	Small "
15	Sprouting green embryos (2 per cent) (reducing sugars present)..................	" "
16	Dry cotyledons (2 per cent) (reducing sugars absent)........................	No "

* Hopkins, C. G., and Pettit, J. H., Soil Fertility Laboratory Manual, Boston, 1910, p. 22.

Hopkins' solution, when used alone with agar gave no growth greater than the control. It has been shown here that a soluble reducing sugar is necessary for growth.

Dry lima bean cotyledons were pulverized and placed in boiling distilled water. This solution plus agar gave no growth. This same solution of the dry cotyledons plus Hopkins' solution gave no growth, but good growth was obtained when 2 per cent glucose was added.

Sucrose plus agar gave good growth, practically the same as glucose.

2 per cent lactose and raffinose gave fair growth.

Mannite gave practically no growth, the leaves turning pale

green and the entire embryo possibly doubling its size in 3 weeks. No lateral roots were formed.

Maltose gave small growth, and soluble starch gave no growth.

Some beans were allowed to sprout in moist sand, the cotyledons turning green. The green cotyledons were macerated and a water extract was made of them. This extract when filtered reduced Fehling's solution, showing the presence of reducing sugars. When this extract was added to agar small growth was obtained.

Some beans were allowed to germinate and sprout for several days on moist cotton. The embryos increased greatly in size and turned green. A water extract of the green sprouting embryos was made which reduced Fehling's solution. An embryo when placed in an agar solution of this extract gave a small growth.

Dry cotyledons of the lima bean, when pulverized and placed in boiling water, do not reduce Fehling's solution and will not support the growth of its own embryo.

SUMMARY.

In these experiments attempts were made to nourish the embryo of the lima bean with different compounds. Glucose and the other sugars have caused growth, while starch or Hopkins' plant food solution failed to cause any appreciable stimulation. Also it has been shown that cotyledons which give no reaction for reducing sugars cause no growth, while these same cotyledons with glucose added give a good growth. It has been shown further that the cotyledons of beans that have germinated and contain reducing sugars also support the growth of an isolated embryo.

In summing up the results, it would appear that where glucose or a carbohydrate giving a hexose on hydrolysis is present, growth is obtained. When these substances are absent no growth results. It is also shown that the dry bean does not contain the plant food necessary for the growth of its own embryo, but that the green cotyledons of a germinated bean do contain the plant food necessary for normal growth.

We hope that other experiments which are now in progress, especially a study of the growth of the isolated embryo of the persimmon seed, will throw more light on this subject.

FETAL ATHYROSIS.

A STUDY OF THE IODINE REQUIREMENT OF THE PREGNANT SOW.

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(Received for publication, November 2, 1916.)

It has been known for a number of years that a large percentage of the sows in some sections of Montana have given birth to hairless and otherwise defective young. Many of these pigs are born dead; others die in an hour or two and very few live more than 24 to 36 hours. The resulting loss in Montana amounts to about 1,000,000 young pigs annually. While the loss is heaviest among swine, there are numerous cases among sheep and occasionally among cattle and horses.

The Affected Districts.

Losses of the same nature have been reported from western North Dakota and South Dakota, Washington,[1] Minnesota, and western Canada. In Montana, the affected area is sharply defined in some instances, less so in others. In many instances the rancher can save his pig crop by moving his pregnant sows a mile or two out of the affected district during the gestation period. Frequently the affected district is a narrow creek bottom, half a mile wide, while the bench land on either side is unaffected. 90 per cent at least of the losses in Montana occur in the drainage system of the Yellowstone River between 109° longitude and the eastern border of the state. This affected area is from 75 to 150 miles wide and is not sharply defined, but all through this region are unaffected spots, a ranch, several ranches, or a whole community, where hairless pigs have never occurred and large numbers of hogs are raised.

[1] *Washington Exp. Station, 25th Ann. Rep.*, 1915, 42.

The Affected Pigs.

The sow carries the affected pigs to the full term of the gestation period. Many ranchers report that the pregnant sows carry the pigs 4 to 7 days longer than the average period. The affected pigs are of full size, occasionally larger than normal. Born of apparently normal sows, they are strikingly weak and low in vitality. The most marked thing in the appearance of a typical specimen is the absence of hair. Except for a few tactile hairs on the nose and a few around the eyes, the skin is smooth, shiny, and bald. The hairless condition is very variable, from almost absolutely hairless to a thin coat, and through all gradations to the normal. The skin, particularly around the shoulders, is thick and feels pulpy. On making an incision the skin is seen to be $\frac{1}{2}$ to $\frac{3}{4}$ of an inch in thickness. It is semitransparent and seems edematous, but no fluid escapes on incision. The hoofs are thin-walled, short, brittle, and plainly in an undeveloped condition. The heart, in every case examined, has a persistent foramen ovale. The thyroid is dark red, sometimes almost black, and presents a most constant enlargement which varies only in proportion to the acuteness of the malady. A histological examination of the thyroid shows a *uniform* hyperplasia and a distention of the blood vessels.

Examination of the Thyroid.

The thyroid was separated from all connective tissue and washed in a gentle stream of water for about 15 minutes; gentle pressure was used to remove as much blood as possible. It was placed between filter papers to remove adhering moisture, and weighed, then cut into fine shreds, dried, and weighed again. The iodine content and the iron content *in the moisture-free* residue were determined, the iodine content by the Hunter method[3] and the *iron content* by filtering the dissolved melt *of the iodine* determination and then estimating the amount of iron, *of the* insoluble residue, by the thiocyanate colorimetric method.

[3] Hunter, A., *J. Biol. Chem.*, 1909–10, vii, 321.

TABLE I.

Thyroid Glands of Unaffected 1 Day Old Pigs.

No.	No. of glands.	Average weight of fresh glands.	Dry matter in fresh glands.	Iodine in desiccated glands.	Iron in desiccated glands.
		gm.	*per cent*	*per cent*	*per cent*
305	44	0.175	20.8	0.095	None.
316	4	0.208	20.9	0.214	"
318	3	0.23	18.4	0.236	"
321	3	0.27	24.2	0.064	"

TABLE II.

Thyroid Glands of Affected Pigs.

No.	Condition.	No. of glands.	Average weight of fresh glands.	Dry matter in fresh glands.	Iodine in desiccated glands.	Iron in desiccated glands.
			gm.	*per cent*	*per cent*	*per cent*
306	Hairless.	36	0.88	17.85	0.0012	0.1
307	Slightly haired, lived 2 days.	2	1.45	16.1	0.0152	0.085
308	Hairless.	3	1.1	18.3	0.0063	0.1
313	Hairless. 6 pigs living in same litter.	1	6.5	15.8	Trace.	0.08
314	"	1	2.9	18.0	0.0001	0.05
315	"	1	1.17	17.7	0.0001	0.05
317	Fairly well haired.	2	0.44	19.7	0.054	None.
319	Hairless.	1	0.64	17.35	0.00864	0.315
320	"	1	3.65	17.5	0.0047	0.15
322	Thin-haired.	1	0.7	22.3	0.015	None.
323	Fairly well haired, lived 3 days, 8 in litter.	2	1.52	15.6	0.00425	"
324	Hairless.	1	0.9	28.05	0.00444	"
325	"	2	1.59	17.55	0.00222	0.125
326	"	4	1.53	18.85	0.009	Trace.
335	"	2	0.61	18.75	0.00525	0.15
336	Skin smooth and shiny, hairless.	8	0.61	19.7	0.003	0.1
337	Hairless. Dark gland.	2	2.6	17.15	0.00135	0.112
338	" Light "	1	3.1	14.0	0.00188	Trace.

The results show that the glands of the affected pigs are abnormally large and the iodine content is extremely low in comparison with those from normal pigs, and that in general the iodine content varies inversely with the degree of hairlessness of the skin. They also show that there is a large accumulation of iron in the glands of the affected pigs. The iron content of the dried blood of the affected pigs averaged 0.2 per cent. If the accumulation of iron was due to an accumulation of a corresponding amount of blood, the greater part of the gland would be composed of blood and there would be a general hemorrhage into the follicles. The histological examination shows that the latter is not probable, and as the greater part of the blood has been removed by the washing of the gland it does not seem possible that the accumulation of iron is due to an accumulation of a corresponding amount of blood.

The Probable Sources of the Malady. A Transmissible Disease (?).

The possibility of contagious abortion was considered. Blood samples were drawn from sows within a week after producing hairless pigs, but no reaction was obtained to the complement fixation test for contagious abortion. Repeated attempts to isolate the bacillus of contagious abortion failed, though material was taken from the placenta and from the stomach contents of the hairless pigs. Further search for an infective agent was not undertaken because the field work strongly indicated that something other than transmissible disease was the cause of the malady. A rancher in one year may lose 95 per cent of his pigs and the next year with the same breeding stock, the same ranch conditions and feed, and water from the same source, raise entirely normal pigs. With the same environment some litters are hairless and others normal; in the same litter are affected and unaffected pigs. In some specific small affected areas, the sows that remain there throughout the gestation period always produce hairless pigs, while those sows that are there for only a part of the gestation period and are then removed to an unaffected area always produce normal pigs. All these facts tend to eliminate transmissible disease as a possible factor.

A Toxic Substance, Present in the Soil, Feed, or Water (?).

A considerable part of the affected areas contains a great deal of alkali, and many stockmen have asserted that alkali was the cause of the malady, but a large number of consistently normal pigs are raised in alkali districts in other parts of the state. Likewise, as alfalfa grows luxuriantly in the affected areas, and in many cases the sows have nothing else to eat, it has been stated that alfalfa would produce hairlessness in young pigs. But in many other sections of the state alfalfa is the principal food for hogs and no hairless pigs occur. In all cases where hairless pigs have been produced, the sow has been, to all appearances, perfectly normal. Can it therefore be due to a toxic substance? Can we conceive of a toxic substance such that when ingested by the mother, it so persistently leaves her to all appearances perfectly normal, yet arrests the development of the fetal growth to such a remarkable extent—one with such a specific action that it only functions in the later stages of the intra-uterine life? The effects do not correspond to the physiological action of any known toxic substance; therefore the probability of a toxic substance being the cause of the malady appears to be very remote.

A Deficiency of Some Essential Constituent.

The apparent symptoms of the affected pigs correspond exactly to the symptoms of myxedema as described by Ord[3] and also to the symptoms occurring after the extirpation of the thyroid as described by the Reverdins,[4] Kocker,[5] and Eiselberg.[6] The hyperplasia of the thyroid varies proportionally with the acuteness of the malady. The results of Hunt and Seidell,[7] Marine,[8] and Marine and Williams[9] show conclusively that the activity of the thyroid depends quantitatively on the iodine content. As

[3] Ord, W. M., *Med.-Chir. Tr.*, 1878, xli, 57.

[4] Reverdin, J. L., and Reverdin, A., *Rev. méd. Suisse romande*, 1883, iii, 169, 233, 309.

[5] Kocker, T., *Arch. klin. Chir.*, 1883, xxix, 254.

[6] von Eiselberg, A., *Arch. klin. Chir.*, 1894, xlix, 207.

[7] Hunt, R., and Seidell, A., *Bull. Hyg. Lab., U. S. P. H.*, 47, 1909.

[8] Marine, D., *Bull. Johns Hopkins Hosp.*, 1907, xviii, 359.

[9] Marine, D., and Williams, W. W., *Arch. Int. Med.*, 1908, i, 378.

the iodine content of the glands of the affected pigs is extremely low, then the activity of thyroid must be correspondingly low. Therefore there seems strong reason to believe that the arrested fetal development of the affected pigs is due to the decreased physiological activity of the thyroid, which the author has designated as fetal athyrosis. On the above considerations an investigation into the iodine requirement of the pregnant sow and a search for the amount of available iodine in the affected districts were undertaken.

A large number of samples of agricultural products from affected and unaffected districts were examined for their iodine content. The results did not indicate anything very definite. Iodine was found in only a few of the samples collected, and in very small amounts. The analyses, however, showed that the amount of available iodine is low and that the average iodine content of samples from affected districts was lower than of samples from unaffected districts. From all results the following has been deduced: While there appears to be enough available iodine to produce the physiologically active constituents of the thyroid required by both young and adult animals, yet, more especially in the affected districts of Montana and to a certain extent over all the western regions, the available iodine is near the border line of the amount required by the pregnant animal. If certain unknown conditions are favorable, the mother may obtain the amount of iodine required for the activities both of its own and fetal glands, but if these conditions are *not favorable*, the maternal organism can still obtain the amount required for the activities of its own thyroid but not enough for the rapidly growing fetus. The iodine starvation of the fetus depresses the physiological activity of the fetal thyroid which causes the remarkably arrested development peculiar to this malady. This is a modification of the contention that has already been brought forward by Fenger[10] after he had examined a great number of adult and fetal glands from animals killed in Chicago. Fenger found that a large percentage of the fetal glands were abnormally large and had a very low iodine content, which he states is not the case with the adult glands. From this he contended that

[10] Fenger, F., *J. Biol. Chem.*, 1913, xiv, 404.

"the demand for iodine in the rapid fetal metabolism and growth in certain instances may exceed the available supply furnished by the pregnant animal. This supply may be sufficient for the maintenance of the maternal metabolism leaving the adult thyroid normal, but not sufficient to prevent iodine starvation and enlargement of the fetal gland."

Iodine Feeding.

Hunt and Seidell[7] say their results "show that when potassium iodide or iodoform is administered to dogs, the thyroids of the latter contain a greatly increased percentage of iodine and also are much more active physiologically."

Marine,[11] in reporting upon glandular hyperplasias of dog thyroids, says that "lack of iodine was the essential deficiency, and iodine when supplied quickly overcame the needs."

Experiment 1.—In the fall of 1915 fifteen sows from an unaffected district were divided into three pens. They were placed on an affected ranch and were bred about December 1, and kept there during the gestation period. They were fed 3 to 5 pounds daily of mixed and red clover hay and sufficient water, all products of the affected ranch. One pen was fed potassium iodide, 15 grains daily per sow, from December 15 to farrowing time; another pen was fed powdered sheep thyroid, and the remainder of the sows were used as a check.

TABLE III.

Pen.	No. of sows.	Special treatment.	No. of young pigs.	Condition of young pigs.	Losses.
1	7	Potassium iodide, 15 grains daily	56	Strong and vigorous.	2
2	5	Sheep thyroid, 5 grains daily................	32	Good...............	6
3	5	No potassium iodide or sheep thyroid........	18	Very poor, weak, dull, and no vitality.............	13

[11] Marine, *J. Infect. Dis.*, 1907, iv, 425.

The owner of the affected ranch added two sows to Pen 1. These had each produced hairless pigs in March and October, 1915. Only four of the sows farrowed in Pen 3 and they produced eighteen pigs. These were weak, wrinkled, squeaking, and puny pigs that appeared to have no chance to thrive; thirteen died in the first few days. They were not hairless but strongly resembled the survivors of a litter which contained hairless pigs. They were thin-haired, had no vitality, and the difference in appearance between these and the pigs of Pen 1 was striking, though the most careful observation could show no material difference between the sows in the two pens.

Experiment 2.—Potassium iodide, in 5 grain daily doses, was fed to twenty-eight sows on five ranches in the affected districts for the last 4 or 5 weeks of the gestation period. The sows that had already farrowed on these ranches had produced hairless pigs. Each of the twenty-eight sows produced normal pigs after receiving potassium iodide.

The object of the above experiments was to demonstrate the difference between the conditions under which hairless pigs are produced and the conditions under which normal pigs are produced. As no hairless pigs were produced in Experiment 1, it appeared, at first, as if the results of that experiment were useless, but there was such a marked difference in the vitality of the young pigs in the respective pens that the results can be considered as conclusive as if hairless pigs had been produced, especially if the intermittent nature of the malady is taken into consideration. The results, besides showing that an abundant supply of iodine counteracts fetal athyrosis, also give a striking demonstration of the beneficial effect of feeding iodine to the pregnant sow.

When iodine is administered as potassium iodide it is quickly detected in the urine but in combination with fats it is retained for a relatively long period in the system; therefore it would probably be more economical to feed iodine as the di-iodostearate obtained by saturating with iodine certain vegetable oils, *viz.*, cottonseed oil, olive oil, etc.

It is intended to recommend the feeding of iodine to the pregnant sows throughout the whole affected areas during the coming season. The wide scope of the test should produce conclusive results.

DISCUSSION.

All the evidence indicates conclusively that the malady is caused by a lack of function of the thyroid. On account of the pronounced hyperplasia of the gland it might be assumed by others that the abnormal growth was produced by some infective agent of low virulence that could not be readily detected, or by some toxic substance, as digitalin, abrin, and ricin are known to produce hyperplasia of the thyroid, and that the beneficial effect of administering potassium iodide was due to the iodide enabling the fetus to resist the infection or neutralizing the action of the toxic substance. It has been pointed out that all evidence tends to disprove these contentions; therefore in the absence of any evidence of the presence of any infective agent or toxic substance, it is most feasible to assume that the cause of the lack of function of the thyroid is due to a deficiency of iodine.

During an investigation of the effect of diet on the activity of the thyroid, Reid Hunt[12] made the hypothesis that certain diets lead to an extensive elimination of iodine of the thyroid. His results indicate that the elimination is greatest with those diets that produce the more rapid growth, eggs, milk, cheese, and various fats.

Fetal athyrosis is prevalent where the sows are fed on alfalfa and flaxseed, and it has occurred, in some cases, where the sows have received large quantities of milk. It is generally recognized that these feeds produce rapid growth and it is probable that they might lead to an abundant secretion of the thyroid and thus exhaust the thyroid of its physiologically active constituents. Therefore, while in the presence of sufficient iodine they may be excellent feeds for the pregnant animal, yet when the amount of available iodine is low they may cause this malady to become more acute. The fact that some of the specimens that were extremely bald were much larger than normal would appear to support this contention.

Seidell and Fenger[13] found that there was a great seasonal variation in the iodine content of the thyroids of sheep, cattle, and hogs killed in Chicago. The iodine content was at a maxi-

[12] Hunt, R., *Bull. Hyg. Lab., U. S. P. H.*, 69, 1910.

[13] Seidell, A., and Fenger, F., *J. Biol. Chem.*, 1912–13, xiii, 517.

mum in the summer, dropped rapidly during the winter, and rose to a maximum again the following summer. Their results show that the iodine content of the glands of hogs is lowest from December to March, the period of the intra-uterine life of the spring litters, and that the yearly iodine curve for hogs is much higher than the curves for sheep and cattle.

It has been noticed that pigs born in March and April are much more frequently affected with fetal athyrosis than those born in May and June, and fall pigs are most frequently normal even in the badly affected districts, which indicates that the seasonal variation exerts a great influence upon the acuteness of this malady, and as the yearly iodine curve for hogs is much higher than the curves for sheep and cattle, a deficiency of the thyroid constituent would cause the offspring of hogs to be more susceptible to fetal athyrosis than those of sheep and cattle. This is in accordance with the well established evidence of the occurrence of the malady.

The results of Martin[14] have shown that composite samples of thyroids, collected throughout the year from sheep slaughtered at Newcastle-on-Tyne, England, varied comparatively little in their iodine content. A striking feature of the results was the uniformly small size of the glands and the relatively high per cent of iodine.

The breeders in Great Britain have won a unique reputation for the excellent livestock that they produce and fetal athyrosis has never been known to occur there. The above results therefore indicate that the shortage of iodine is more acute in North America than in Great Britain. When all the results are correlated it appears feasible to assume that if the pregnant animals on this continent were given more iodine, especially during the winter months, the young that they produce would be more healthy and more vigorous and the number of weak and defective young animals that are produced annually would be greatly reduced.

It has been generally contended by others that the lack of growth of the epidermal appendages in myxedema and after extirpation of the thyroid does not indicate a direct relation

[14] Martin, N. H., *Brit. and Colonial Druggist*, 1912, lxii, 99.

between the thyroid and the production of those appendages, but is due to inhibition by the toxic substances of metabolism which have not been neutralized by secretion of the thyroid. There is every evidence that the development of the epidermal appendages is arrested, in fetal athyrosis, in the later stages of the intra-uterine life when the fetal blood is oxygenated by the maternal organism, and it can be assumed that any toxic substance in the fetal blood would be neutralized at the same time by the maternal organism. Therefore it does not seem possible that the lack of growth of the epidermal appendages as found in fetal athyrosis and probably also after extirpation of the thyroid and with myxedema is due to inhibition by toxic substances of metabolism and growth. While it may be feasible to assume that the maternal organism would neutralize the toxic substances of the fetal blood, it is not probable that the maternal thyroid could give the required assistance for the proper development of the fetus.

It has been pointed out that the thyroids of the affected pigs are a very dark red color and that there is an accumulation of iron in the gland. This accumulation of iron is significant as myxedema is accompanied by anemia and after extirpation of the thyroid there is a rapid decrease of red corpuscles.

CONCLUSIONS.

An iodine deficiency during the gestation period causes a lack of function and hyperplasia of the fetal thyroid, resulting in an arrested development of the fetus.

If more iodine were fed to the pregnant animals in large sections of this continent, especially during the winter months, the young that they produce would be more healthy and more vigorous and the large number of weak and defective young animals that are produced annually would be greatly reduced.

Fetal athyrosis presents strong evidence that there is a direct relation between the physiologically active constituents of the thyroid and growth of the epidermal appendages.

An abundant secretion of the fetal thyroid, during the later stages of the intra-uterine life, is essential for the normal development of the fetus.

STUDIES IN CARBOHYDRATE METABOLISM.

XIV. THE INFLUENCE OF ALKALI ADMINISTRATION UPON BLOOD SUGAR CONTENT IN RELATION TO THE ACID-BASE-PRODUCING PROPERTIES OF THE DIET.*

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The influence of acid and alkali upon carbohydrate metabolism with respect to glycosuria and blood sugar content has recently received considerable attention. In a discussion of the subject, it seems desirable to differentiate between the results obtained by administration of acid or alkali to normal animals and those yielded when the sugar-regulating mechanism has been temporarily disturbed.

Elias demonstrated that the introduction of hydrochloric acid (0.25 N) into dogs and rabbits leads to hyperglycemia and glycosuria. Underhill (1916, *a*) found that the subcutaneous injection of calcium salts (lactate and chloride), which under disturbed conditions of carbohydrate metabolism have an effect analogous to hydrochloric acid, is without significant influence upon the blood sugar content of normal rabbits; and that the withdrawal of calcium by the introduction of sodium phosphate will usually, although not invariably, produce in normal rabbits a condition of hypoglycemia. This investigator showed also (1916, *b*) that the intravenous injection of sodium carbonate into normal animals may induce a decided though transient fall in blood sugar content. Marked hypoglycemia is indicated in slightly more than one-half the instances reported.

Recently Macleod and Fulk found that the injection of sodium carbonate solution or of hydrochloric acid, at intervals, into the portal vein during the continuous injection of a glucose solution into another branch of the same vein has no effect upon the blood sugar. But when glucose is injected at a rate below the tolerance limit with sufficient alkali to reduce distinctly the hydrogen ion concentration of the blood of the portal vein and vena cava, the blood sugar does not rise to such a degree as it does when the glucose solution is neutral or acid in reaction.

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* The data in this and the six papers following are taken from the dissertation presented by Louise McDanell for the degree of Doctor of Philosophy, Yale University, 1917.

The influence of the introduction of acid or alkali into animals with a defective sugar-regulating mechanism has been studied more extensively. Pavy and Godden gave intravenously a 3 per cent solution of anhydrous sodium carbonate to cats exhibiting glycosuria in consequence of the administration of chloroform. They observed a fall in the intensity of the glycosuria within 15 minutes after the injection was begun, the urine becoming sugar-free at the end of 30 minutes. It was suggested by them that the inhalation of chloroform may produce a condition of acidosis which leads to an abnormal conversion of glycogen into sugar, and hence to glycosuria. Elias and Kolb studied the influence of sodium carbonate upon glycosuria, hyperglycemia, and sugar tolerance in the "hunger diabetes" of young dogs, and found that the alkali reduces the glycosuria and glycemia. That this "hunger diabetes" is a condition of acidosis is indicated by the lowered carbon dioxide content of the blood and alveolar air. Murlin and Kramer (1913) showed that after removal of the pancreas, sodium carbonate introduced into the blood stream of the dog causes a diminution of the sugar excretion. Later experiments by the same authors (1916) indicated: first, that sodium bicarbonate and potassium bicarbonate administered by stomach tube may be without immediate effect upon the glycosuria and hyperglycemia of the depancreatized dog; second, that an alkali bicarbonate given by mouth to a fasting depancreatized dog may even cause the reappearance of glucose in the urine after it has been "starved out;" third, that the normal anhydrous salt of sodium, Na_2CO_3, may, on the contrary, reduce the sugar in the urine materially, when given by mouth, and when given by vein invariably does so, especially when added to Ringer's or Locke's solution to the amount of about 1 per cent; and fourth, that dilute hydrochloric acid given by mouth or subcutaneously to the depancreatized dog has an effect opposite to that of alkali, increasing the sugar in the urine without affecting materially the nitrogen elimination, and without causing any effect upon the blood sugar.

It was shown by Underhill and Blatherwick that in thyreoparathyroidectomized animals, the blood sugar content may be greatly diminished previous to the onset of tetany. At this period, Wilson, Stearns, and Thurlow, and Wilson, Stearns, and Janney found that a condition of alkalosis exists, the duration of which corresponds fairly well with the interval of low blood sugar observed by Underhill and Blatherwick. The tetany is relieved and the blood sugar restored to normal by the introduction of calcium. The intravenous administration of hydrochloric acid relieves the tetany, but apparently its effect upon the blood sugar has not been investigated. It seems probable that the condition of alkalosis may be responsible for the hypoglycemia.

It was first shown by Underhill (1911) that the subcutaneous injection of hydrazine sulfate invariably leads to hypoglycemia in dogs, and frequently in rabbits. Later MacAdam called attention to the fact that, provided the dose of hydrazine is sufficiently large, rabbits also always exhibit hypoglycemia. Underhill and Baumann recently demonstrated

that, in the dog, during this period of hypoglycemia, the hydrogen ion concentration of the urine is greatly depressed, even to the point of marked alkalinity.

Underhill (1916, *b*) found that sodium carbonate, if administered at suitable intervals previous to epinephrine, significantly reduces the hyperglycemia and glycosuria incident to injections of that drug. Calcium, which Wilson and his coworkers showed to exert an acid-like action at times in the body, exhibits (Underhill, 1916, *a*) an effect opposite to that of sodium carbonate, causing an increased elimination of sugar in the urine, and altering the character of the curve of epinephrine hyperglycemia. The withdrawal of calcium, by the injection of sodium phosphate prior to the administration of epinephrine, reduces the sugar level and shortens the period of hyperglycemia, and frequently diminishes the output of sugar in the urine, in comparison with conditions brought about by epinephrine alone. It has been shown by Macleod and Fulk that there is a distinct decrease in the blood sugar in animals in which there is hyperglycemia (etherization and operative manipulation in sugar-fed dogs), when alkali (Na_2CO_3) is injected intravenously in sufficient amount to lower the hydrogen ion concentration of the blood. Underhill (1916, *c*) reported the disappearance of glycosuria in a very severe case of human diabetes, following the ingestion of large doses of sodium bicarbonate.

The experiments detailed in the present paper were undertaken for the purpose of determining the factors responsible for the inconstant production of hypoglycemia in normal rabbits after the intravenous injection of sodium carbonate, as reported by Underhill (1916, *b*). The significance of the diet, with respect to its potential acid- or base-producing properties, has received special attention.

Methods.

The experiments were performed upon healthy, full-grown rabbits. Blood was obtained from the ear vein, the sample weighed, and the blood sugar estimated by the method of Lewis and Benedict.[1, 2] After a sample of blood had been taken for the normal blood sugar, sodium carbonate in 0.5 per cent solution was introduced, under pressure, into the ear vein, 2 to 5 minutes being required for the injection. Samples of blood for the determination of sugar were taken 30, 60, and 90 minutes thereafter. Allowance was not made for dilution of the blood, as other experiments in this laboratory[3] have demonstrated that,

[1] McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.

[2] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

[3] Bogert, L. J., Underhill, F. P., and Mendel, L. B., *Am. J. Physiol.*, 1916, xli, 189.

under the conditions here maintained, the blood volume returns to normal in less than 30 minutes, the interval allowed before the first sample was taken.

No difficulty was experienced in these experiments, or elsewhere, on account of the "emotional glycosuria" described by Cannon, Shohl, and Wright.[4] Allen[5] accounts for the variations in the blood sugar of the rabbit, as given by various authors, by the statement: "The rabbit is an animal subject to emotional and traumatic hyperglycemia." Later he explains the apparent low tolerance of the rabbit for glucose, in some instances, by saying: "Traumatic, emotional, or other disturbing influences are probably the explanation, in a highly erratic and uncertain animal." The experience in this laboratory, where many rabbits are used, leads to an opposite conclusion. Rabbits are usually quiet, docile, and seemingly unconcerned about any ordinary treatment accorded them. Even when apparently excited, there has been no noticeable effect upon the blood sugar.

The diets of these rabbits were the same as those used by Underhill[6]—whole oats and cracked corn for the acid-forming diet, carrots for the base-forming diet, and a mixture of these for the "mixed" diet.

The results are shown in Tables I, II, and III. From these it may be seen that, with the single exception of Rabbit 17, there was no marked diminution of the blood sugar following the injection of alkali. The initial blood sugar of Rabbit 17 was much higher than that of any other rabbit which was used, and, after the drop of one-third in the first half hour, it was still above our average for normal rabbits. The further wide variations in the blood sugar in subsequent periods would tend to confirm the belief that Rabbit 17 is not to be considered a normal animal. While there are a few instances of an appreciable augmentation of blood sugar content following the injection of the sodium carbonate (Rabbits 18, 19, 22), the increase is too slight to be attributable to the alkali, in view of the large number of cases which showed no significant change. The hyperglycemia may be

[4] Cannon, W. B., Shohl, A. T., and Wright, W. S., *Am. J. Physiol.*, 1911–12, xxix, 280.

[5] Allen, F. M., Studies concerning *Glycosuria and Diabetes*, Boston, 1913.

[6] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

accounted for in Rabbit 22 by the prostration incident to the injection. The few cases of prostration in our experience have all been accompanied by a marked hyperglycemia.

The data presented indicate that the injection of sodium carbonate is as ineffective in altering the blood sugar content of normal rabbits maintained upon either acid-forming or base-forming diets as it is in animals upon a mixed diet.

TABLE I.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon a Mixed Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	*cc.*				
March 7.....	15	2,200	125	0.13	0.132	0.14	0.122
" 7.....	16	2,060	125	0.132	0.133	0.13	0.133
" 11.....	17	1,940	125	0.18	0.12	0.176	0.115
" 11.....	18	1,980	125	0.14	0.20	0.188	0.19
" 13.....	19	2,800	125	0.12	0.149	0.118	0.144
" 13.....	20	2,000	100	0.11	0.12	0.12	0.117
" 15.....	21	2,360	125	0.119	0.12	0.115	0.124
" 15.....	22*	2,480	120	0.116	0.154	0.164	0.142
" 17.....	23	2,680	140	0.11	0.117	0.137	0.136
" 17.....	24	2,300	125	0.131	0.124	0.12	0.143

* Prostrated by injection.

TABLE II.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon an Acid-Forming Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	*cc*				
May 3........	27	1,420	120	0.119	0.137	0.15	0.139
" 3........	29	1,900	120	0.115	0.13	0.13	0.127
" 5........	30	1,660	123	0.11	0.117	0.119	0.123
" 5........	31	1,760	110	0.117	0.13	0.13	0.14

TABLE III.

The Influence of Intravenous Injections of 0.5 per Cent Sodium Carbonate upon the Blood Sugar Content of Rabbits Maintained upon a Base-Forming Diet.

Date.	Rabbit.	Weight.	Volume of sodium carbonate injected.	Blood sugar (in percentages).			
				Normal.	Min. after injection.		
					30	60	90
		gm.	cc.				
May 10.......	29	2,000	123	0.102	0.115	0.11	0.11
" 10.......	32	1,500	120	0.12	0.112	0.126	0.135
" 11.......	33	2,080	120	0.104	0.11	0.115	0.12
" 11.......	34	1,760	120	0.113	0.107	0.107	0.109
" 12.......	35	2,140	120	0.104	0.104	0.113	0.111
" 12.......	36	1,540	60	0.11	0.115	0.11	0.114

CONCLUSIONS.

In new experiments upon the influence of sodium carbonate on the blood sugar content of normal rabbits on a mixed diet, hypoglycemia has invariably failed to manifest itself.

The variable potency of sodium carbonate in the production of hypoglycemia, as reported by Underhill, cannot be explained by variations in the acid-base-producing possibilities of the diet, inasmuch as wide ranges in these directions apparently play a small part in influencing the blood sugar content of normal rabbits.

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STUDIES IN CARBOHYDRATE METABOLISM.

XV. THE INFLUENCE OF ACID-FORMING AND BASE-FORMING DIETS UPON BLOOD SUGAR CONTENT.

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As the result of a study, by Sherman and Gettler, of the ash of foods, emphasis has been laid upon the striking differences of foodstuffs with respect to their property of yielding an ash with a preponderance of either acid or basic elements. They demonstrated that fruits and vegetables yield a basic ash, and that cereals, meat, and fish give an acid ash. It was also pointed out by these investigators that the acid-forming or base-forming character of ingested food plays a significant rôle in the regulation of urinary composition. Thus, they found an augmentation in the ammonia excretion equivalent to as much as one-third of the increased potential acid in the diet. A still greater proportion of this acid was represented by the increased acidity of the urine.

Blatherwick extended the work of Sherman and Gettler with respect to the relation of the urinary reaction to the character of the diet. He found that in general the reaction of the urine varies with that of the ash constituents of the diet. Exceptions to the rule were noted with plums, prunes, and cranberries, which yield an acid urine. The cause for this unexpected result is ascribed to their content of benzoic acid, which is incompletely oxidized in the body and excreted as hippuric acid. Underhill, in a study of creatine metabolism, showed that in rabbits, which normally excrete an alkaline urine, a diet of oats and cracked corn gives rise to a urine of high acidity, while a diet of carrots increases the alkalinity beyond the normal.

In an investigation of the influence of acid and alkali upon carbohydrate metabolism, it was deemed desirable to determine whether any influence would be exerted upon blood sugar content by differences in the acid-base character of the diet. The detailed experiments follow.

Methods.

The experiments were made upon normal, full-grown rabbits. The diets were the same as those used by Underhill—oats and cracked corn for the acid-forming diet, carrots for the base-forming diet, and a mixture of these for the "mixed" diet. Water was offered several times a day. Blood sugar was determined, usually in the morning and afternoon, according to the method of Lewis and Benedict.[1,2] For the estimation of hydrogen ion concentration in the urine, the procedure of Henderson and Palmer[3] was employed, and the results are expressed in terms of the logarithmic notation of Sörensen used by them. On account of the strong alkalinity of the urine from the carrot and mixed diets, hydrogen ion concentration was determined only during the periods of the grain diet.

Tables I and II show the results for rabbits which were under observation for about 6 weeks. The results for rabbits for which the experimental period was shorter are given in Tables III to VI.

The most striking fact brought out by these data is the constancy of the values for blood sugar, notwithstanding the wide variations in the hydrogen ion concentration of the urine. Evidently the sugar-regulating mechanism of a normal rabbit is not significantly disturbed by such variations in acidity as occurred here; or, expressed differently, its power of compensation is sufficiently great to enable it to handle these amounts of acid without interference with the acid-base equilibrium of its body.

Attention may be called to the cases of greatest variation in the blood sugar content. Rabbit 25 showed a blood sugar value of 0.085 per cent on March 27 and of 0.084 per cent on April 5, while with Rabbit 26 there was a value of 0.085 per cent on March 27, and of 0.076 per cent on April 6. These low values occurred during the acid-forming diet, only a small portion of which was eaten, and also after the rabbits, both of which were small, had lost considerable weight. The cases of highest blood sugar, Rabbit 26, 0.161 per cent on April 24, and Rabbit 30, 0.162 per cent on April 19, as well as other high values, were obtained upon

1 Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.

2 McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.

3 Henderson, L. J., and Palmer, W. W., *J. Biol. Chem.*, 1912–13, xiii, 393.

the base-forming rations. This diet the rabbits relished; usually they ate all that was given them, and upon it maintained their weight or even gained. Other experiments in this series[4] show that rabbits store very little glycogen upon an acid-forming diet when the intake is as small as it was with the animals under discussion. It is therefore possible that the variations noted in the blood sugar content may be related to dietary factors other than those concerned with the acid-base properties.

TABLE I.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 25.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Mixed diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
March 20...		1,700	0.119	0.135		
" 21...			0.122	0.104	75	Alkaline.
" 22...	Grain 30 Carrots 125		0.108	0.104	44	"
" 23...	Grain 39 Carrots 120		0.098	0.114	45	"
Acid-forming diet.†						
March 24...	40	1,600	0.114	0.121	18	Alkaline.
" 25...	35		0.123		25	"
" 26...	30		0.10		25	"
" 27...	40	1,500	0.089	0.085	25	"
Base-forming diet.‡						
March 28...	430		0.122	0.13	90	Alkaline.
" 29...	500	1,500	0.129	0.125	275	"
" 30	500		0.113		375	"
" 31...	500	1,400	0.121	0.116	385	"

* Corn and oats, 50 gm., carrots, 250 gm.

† Corn and oats, 100 gm.

‡ Carrots, 500 gm.

[4] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 255.

TABLE I—*Concluded.*

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Acid-forming diet.†						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 1.....	57			0.114	15	Alkaline.
" 2.....	40	1,400	0.119		25	
						H ion.
" 3.....	52		0.111	0.117	15	5.5
" 4.....	43	1,440	0.092	0.098	20	5.7
" 5.....	55		0.105	0.084	20	5.7
" 6.....	55	1,460	0.10	0.106	20	5.7
" 7.....	60		0.10	0.098	10	5.5
" 8.....	57	1,440	0.102	0.087	30	5.8
" 9.....	60		0.097		25	5.3
" 10....		1,440	0.106	0.098	50	5.8
Base-forming diet.‡						
						H ion.
April 11.....	488		0.113	0.10	115	6.7
" 12.....	500	1,500	0.096	0.101	260	6.9
" 13.....	500		0.099		285	7.38
" 14.....	500	1,460	0.109	0.11	260	Alkaline.
" 15.	500		0.126	0.113	330	"
" 16.....	500	1,460	0.115		315	"
" 17.....	455		0.132	0.113	245	"
" 18.....	500	1,500	0.107	0.106	360	"
" 19.....	500		0.107	0.118	245	"
" 20.....	500	1,520	0.116	0.101	285	"
" 21.....	500		0.11	0.116	265	"
" 22.....	500	1,520	0.106	0.121	325	"
" 23.	500		0.124		365	"
" 24.....	500		0.133	0.135	320	"
Acid-forming diet.†						
April 25.....	52		0.117	0.123	75	Alkaline.
						H ion.
" 26.....	50	1,520	0.097	0.106	45	5.3
" 27.....	30		0.116		36	4.9
" 28.....	25	1,500	0.112	0.119	26	4.7
" 29.....	15		0.118	0 097	25	5.3
" 30.....	10	1,440	0.113		15	5.3

TABLE II.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content.
Rabbit 26.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine	
			A m.	P.m	Volume.	Reaction to litmus
Mixed diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc*	
March 20...		2,000	0.128	0 119		
" 21...			0.128	0 108	140	Alkaline.
" 22...			0.115	0.112	108	"
" 23...	Grain 50 Carrots 250		0.121		108	"
Acid-forming diet.†						
March 24...	55	1,840	0.11	0.115	64	Alkaline.
" 25...	52		0.108		80	"
						H ion.
" 26...	10		0.097		130	7 38
" 27...	8	1,560	0 085	0 098	155	7.43
Base-forming diet.‡						
March 28...	410		0 121	0 123	230	Alkaline.
" 29...	500	1,720	0.129	0 121	385	"
" 30...	500		0.115		315	"
" 31...	500	1,720	0.12	0 11	400	"
Acid-forming diet.†						
April 1.....	50			0 121	110	Alkaline.
" 2.....	60	1,680	0 117		95	
						H ion.
" 3.....	46		0.129	0 103	80	6 7
" 4.....	60	1,660	0 128	0 123	75	6 5
" 5.....	53		0 117	0 092	80	6 8
" 6.....	45	1,640	0 076	0 093	60	6 3
" 7.....	41		0 108	0 103	55	5 7
" 8.....		1,600	0 111	0 117	55	5 7
" 9.....	40		0 097		60	5 5
" 10.....			0 104	0 099	45	5 3

* Corn and oats, 50 gm.; carrots, 250 gm.
† Corn and oats, 100 gm.
‡ Carrots, 500 gm.

TABLE II—*Concluded.*

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.‡						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 11.....	500		0.095	0.10	175	6.8
" 12.....	500	1,620	0.107	0.093	400	7.38
" 13.....	500		0.112	0.119	285	Alkaline.
" 14.....	500	1,680	0.108	0.106	385	"
" 15.....	500		0.113	0.148	320	"
" 16.....	500	1,660	0.107		505	"
" 17.....	500		0.109	0.136	305	"
" 18.....	500	1,800	0.098	0.127	445	"
" 19.....	500		0.113	0.126	420	"
" 20.....	500	1,880	0.133	0.132	275	"
" 21.....	500		0.129	0.158	285	"
" 22.....	600	1,800	0.134	0.128	395	"
" 23.....	500		0.132		360	"
" 24.....	500		0.114	0.161	450	"
Acid-forming diet.†						
April 25.....	80		0.114	0.133	95	Alkaline.
" 26.....	93	1,780	0.124	0.111	38	
						H ion.
" 27.....	63		0.13		35	4.7
" 28.....		1,760	0.12	0.112		
" 29.....	60		0.122	0.128	36	4.7
" 30.....		1,740	0.138			

TABLE III.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 27.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar. A.m.	Blood sugar. P.m.	Urine. Volume.	Urine. Reaction to litmus.
Base-forming diet.*						
	gm.	gm.	per cent	per cent	cc.	
April 14.....		1,820				
" 15.....	460		0.114	0.104	210	Alkaline.
" 16.....	500	1,900	0.112		325	"
" 17.....	500		0.114	0.102	320	"
" 18.....	500	1,900	0.098	0.094	345	"
" 19.....	500		0.114	0.117	250	"
" 20.....	500	1,860	0.106	0.116	295	"
" 21.....	500		0.124	0.107	255	"
" 22.....	410	1,800	0.113	0.11	295	"
" 23.....	500		0.116		300	"
" 24.....	440	1,780	0.11	0.123	280	"
Acid-forming diet.†						
April 25.....			0.115	0.099	55	Alkaline.
						H ion.
" 26.....	33	1,640	0.11	0.10	29	5.85
" 27.....	42		0.12		35	5.3
" 28.....		1,620	0.134	0.107		
" 29.....	60		0.114	0.095	56	5.7
" 30.....	20	1,540	0.118		40	4.7
May 1.....	5		0.13		35	4.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE IV.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 29.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	gm.	per cent	per cent	cc.	
April 14.....		1,980				
" 15.....	402		0.117	0.11	240	Alkaline.
" 16.....	500	2,020	0.108		245	"
" 17.....	500		0.109	0.096	325	"
" 18.....	500	2,100	0.108	0.11	340	"
" 19.....	500		0.127		365	"
" 20.....	500	2,000	0.118	0.106	375	"
" 21.....	500		0.133	0.11	280	"
" 22.....	500	2,080	0.118	0.114	280	"
" 23.....	500		0.123		420	"
" 24.....	500	2,020	0.127	0.127	270	"
Acid-forming diet.†						
April 25.....	80		0.116	0.112	45	Alkaline.
						H ion.
" 26.....	90	1,960	0.115	0.104	30	6.3
" 27.....	65		0.11		15	5.85
" 28.....	65	2,000	0.11	0.104	20	5.7
" 29.....	40		0.106	0.106	29	5.85
" 30.....	23	1,940	0.116		26	5.7
May 1.....	20		0.119		22	5.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE V.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 30.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar. A.m.	Blood sugar. P.m.	Urine. Volume.	Urine. Reaction to litmus.
			Base-forming diet.*			
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 14.....		1,920				
" 15.....	500		0.136	0.106	350	Alkaline.
" 16.....	500	1,820	0.132		400	"
" 17.....	500		0.123	0.123	310	"
" 18.....	500	1,920	0.135	0.125	340	"
" 19.....	500		0.162	0.137	210	"
" 20.....	500	1,980	0.143	0.123	310	"
" 21.....	500		0.125	0.123	370	"
" 22.....	500	1,880	0.125	0.125	390	"
" 23.....	500		0.128		405	"
" 24.....	500	1,820	0.108	0.10	400	"
			Acid-forming diet.†			
April 25.....	76		0.118	0.116	55	Alkaline.
						H ion.
" 26.....	75	1,800	0.11	0.127	45	5.1
" 27.....	50		0.105		55	4.7
" 28.....	45	1,720	0.106	0.10	44	4.7
" 29.....	18		0.107	0.102	30	4.7
" 30.....	30	1,680	0.108		35	4.7
May 1.....	45		0.10		34	4.9
" 2.....	55	1,720	0.107	0.09	30	5.1
" 3.....	57		0.11		40	4.9
" 4.....	50		0.109		22	5.1
" 5.....	28	1,660	0.11		27	4.9

* Carrots, 500 gm.
† Corn and oats, 100 gm.

TABLE VI.

The Effect of Changes in the Character of the Diet upon Blood Sugar Content. Rabbit 31.

Date.	Weight of food eaten.	Weight of rabbit.	Blood sugar.		Urine.	
			A.m.	P.m.	Volume.	Reaction to litmus.
Base-forming diet.*						
	gm.	*gm.*	*per cent*	*per cent*	*cc.*	
April 16.....	500	2,040	0.102		290	Alkaline.
" 17.....	500		0.109	0.105	370	"
" 18.....	500	2,040	0.105	0.103	390	"
" 19.....	500		0.107	0.112	275	"
" 20.....	500	1,960	0.127	0.115	370	"
" 21.....	500		0.119	0.118	290	"
" 22.....	500	2,020	0.12	0.111	370	"
" 23.....	500		0.111		320	"
" 24.....	500	2,060	0.114	0.123	370	"
Acid-forming diet.†						
April 25.....	75		0.125	0.099	85	Alkaline.
						H ion.
" 26.....		2,080	0.109	0.101	85	6.3
" 27.....	138		0.106		8	6.9
" 28.....	35	2,040	0.11	0.098	105	5.3
" 29.....	28		0.112	0.107	125	5.3
" 30.....	33	1,940	0.105		78	4.7
May 1.....	35		0.109		90	4.7
" 2.....	18	1,840	0.117		120	4.7
" 3.....	60			0.114	55	4.7
" 4	5		0.128		67	4.7
" 5.....	20	1,760	0.117		75	4.7

* Carrots, 500 gm.

† Corn and oats, 100 gm.

TABLE VII.

The Average Figures for Blood Sugar Content of Rabbits Maintained upon Different Diets.

Rabbit.	Acid-forming diet.	Base-forming diet.
	per cent	*per cent*
25 Period 1	0.105	0.122
" 2	0.102	0.113
" 3	0.111	
26 Period 1	0.102	0.12
" 2	0.108	0.121
" 3	0.123	
27	0.112	0.111
29	0.111	0.115
30	0.108	0.127
31	0.11	0.113
General averages	0.109	0.117

CONCLUSIONS.

In a normal rabbit the blood sugar content is not significantly changed by variations in the acid-base content of the diet, which are sufficient to cause a marked change in the hydrogen ion concentration of the urine. These results are in agreement with those of the preceding paper, which indicate that the blood sugar content of a normal rabbit is not usually appreciably affected by intravenous injections of dilute alkali.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVI. THE RELATION OF EPINEPHRINE GLYCOSURIA TO DOSAGE AND TO THE CHARACTER OF THE DIET.

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Recently it was observed by Underhill (1916) that the glycosuria (and hyperglycemia) produced by subcutaneous injections of epinephrine is decreased by the intravenous injection of alkali (sodium carbonate, in 0.5 per cent solution). Here Underhill used doses of 1 mg. of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000) per kilo of body weight. In planning to extend this work, it was considered possible that the effect of the alkali in reducing the glycosuria and hyperglycemia might be more marked if a minimum effective dose of epinephrine were administered. Accordingly, the relation of glycosuria to variations in the quantity of epinephrine administered has been determined. The influence of acid-forming and base-forming diets upon the glycosuria brought about by given doses of epinephrine has also been investigated.

Methods.

Eighteen full-grown rabbits were divided into three groups, A, B, and C, each group being fed for 1 week with (1) a mixed diet of oats, cracked corn, bread, carrots, celery, grass; (2) a base-forming diet of carrots and celery; (3) an acid-forming diet of oats and cracked corn; (4) a mixed diet similar to that of the first week. A surplus of the mixed and grain diets was always available, while the base-producing food was usually entirely consumed. An abundance of water was supplied. Epinephrine (adrenalin chloride, 1: 1,000, Parke, Davis and Company) was administered in doses of 0.1 to 0.5 mg. per kilo of body weight.

TABLE I.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group A.

Rabbit.	32	33	34	35	36	29	50
Epinephrine per kilo, mg.	0.5	0.4	0.3	0.2	0.3	0.2	0.2
May 15–16. Base-forming diet.							
Weight, gm.	1,460	2,040	1,700	1,940	1,500	2,140	
Urine volume, cc.	175	270	115	70	230	210	
Glucose, gm.	0.163	0.0763	0.0		0.01	0.023	
May 23–24. Mixed diet.							
Weight, gm.	1,540	2,100	1,660	2,120	1,520	2,260	
Urine volume, cc.	.130	170	155	155	75	145	
Glucose, gm.	0.663	0.097	0.112	0.184	1.424	0.398	
May 29–30. Acid-forming diet.							
Weight, gm.	1,520		1,580	1,900	1,540	2,080	1,820*
Urine volume, cc.	100		50	50	60	80	105
Glucose, gm.	0.139		0.042	0.065	0.559	0.278	0.017
June 5–6. Mixed diet.							
Weight, gm.	1,480		1,700	2,160	1,520	2,040	1,800
Urine volume, cc.	125		240	170	55	160	105
Glucose, gm.	0.476		0.000	0.101	0.767	1.015	0.012

* Mixed.

TABLE II.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group B.

Rabbit.	37	38	39	40	41	42	51
Epinephrine per kilo, mg.	0.4	0.4	0.3	0.3	0.2	0.2	0.2
May 17–18. Mixed diet.							
Weight, gm.	1,460	2,060	1,380	2,440	1,600	1,400	
Urine volume, cc.	105	150	190	325	90	60	
Glucose, gm.	1.887	1.807	0.103	3.456	0.289	0.562	
May 25–26. Base-forming diet.							
Weight, gm.	1,540	2,160	1,420	2,600	1,460	1,220	
Urine volume, cc.	205	325	240	350	210	230	
Glucose, gm.	0.807	1.764	0.789	2.079	0.397	0.065	
June 1–2. Acid-forming diet.							
Weight, gm.	1,540	2,020	1,400	2,060	1,460		1,780*
Urine volume, cc.	80	80	15	75	55		75
Glucose, gm.	1.076	0.724	0.344	0.333	0.055		0.026
June 8–9. Mixed diet.							
Weight, gm.	1,540	2,160	1,460	2,460	1,620		1,700
Urine volume, cc.	140	95	85	225	105		60
Glucose, gm.	1.77	1.045	1.120	3.915	0.134		0.65

* Mixed.

At the end of each week, each rabbit was given subcutaneously the specified dose of epinephrine and placed in a metabolism cage for 24 hours. Sugar in the urine was determined gravimetrically according to Allihn's method, the copper being weighed as cupric oxide.

TABLE III.

The Relation of Epinephrine Glycosuria to Dosage and to the Character of the Diet. Group C.

Rabbit.	43	44	45	46	47	48	49
Epinephrine per kilo, mg.	0.3	0.3	0.2	0.2	0.1	0.1	0.1
May 19–20. Mixed diet.							
Weight, gm................	1,640	2,720	2,660	1,680	1,880	2,500	
Urine volume, cc..........	75	175	155	50	140	160	
Glucose, gm................	0.015	4.518	1.697	0.023	0.000	0.161	
May 26–27. Base-forming diet.							
Weight, gm................	1,500	2,260	2,600	1,680	1,600		1,640*
Urine volume, cc..........	350	115	250	240	240		110
Glucose, gm................	1.474	2.146	0.124	0.49	0.173		
June 2–3. Acid-forming diet.						No. 52*	
Weight, gm................		1,980	2,600	1,760	1,740	2,140	1,420
Urine volume, cc..........		130	115	45	45	60	50
Glucose, gm................		3.480	0.815	0.074	0.063	1.039	0.022
June 9–10. Mixed diet.						No. 53*	
Weight, gm..		2,080	2,580	1,620	1,740	2,740	1,480
Urine volume, cc		125	155	75	35	130	45
Glucose, gm................		2.720	1.400	0.150	0.000	3.583	0.030

* Mixed.

The Relation of Glycosuria to Variations in the Quantity of Epinephrine Administered.

From Tables IV and V, arranged according to the dosage, it may be seen that 0.4 mg. of epinephrine per kilo always induced glycosuria; that 0.3 mg. per kilo caused glycosuria in every instance except two (Rabbit 34), and that the average excretion of sugar incident to this dose was greater than that following the 0.4 mg. dose, although the small number of rabbits receiving the latter amount reduces the value of this comparison. With a dose of 0.2 mg. of epinephrine per kilo, glycosuria resulted in every instance, although the average amount of sugar eliminated

was less than one-third of that excreted after a dose of 0.3 mg. per kilo. A dose of 0.1 mg. of epinephrine per kilo was given only to three animals (Rabbits 47, 48, and 49, Table III). This

TABLE IV.

The Output of Sugar in the Urine Following Varying Doses of Epinephrine, in Relation to Changes in the Character of the Diet.

Rabbit.	Epinephrine per kilo.	Glucose in urine with			
		Mixed diet.	Basic diet.	Acid diet.	Mixed diet.
	mg.	*gm.*	*gm.*	*gm.*	*gm.*
33	0.4	0.097	0.076		
37	0.4	1.887	0.807	1.076	1.77
38	0.4	1.807	1.764	0.724	1.045
Average..........	....	1.264	0.882	0.900	1.407
34	0.3	0.112	0.000	0.042	0.000
36	0.3	1.424	0.01	0.559	0.767
39	0.3	0.103	0.789	0.344	1.120
40	0.3	3.456	2.079	0.333	3.915
43	0.3	0.015	1.474		
44	0.3	4.518	2.146	3.480	2.720
52	0.3				1.039
53	0.3				3.583
Average..........	....	1.605	1.083	0.952	1.878
35	0.2	0.184		0.065	0.101
29	0.2	0.398	0.023	0.278	1.015
41	0.2	0.289	0.397	0.055	0.134
42	0.2	0.562	0.065		
45	0.2	1.697	0.124	0.815	1.400
46	0.2	0.023	0.49	0.074	0.150
50	0.2				0.017
50	0.2				0.012
51	0.2				0.026
51	0.2				0.650
Average..........	....	0.626	0.22	0.257	0.389
General average..	....	1.105	0.732	0.654	1.081

amount failed to produce glycosuria in two cases out of seven. In view of these results it seems probable that a dose of 0.3 mg. of epinephrine per kilo of body weight may be relied upon to induce glycosuria when the liver contains a sufficiency of glycogen.

TABLE V.

The Output of Sugar in the Urine Following Varying Doses of Epinephrine, in Relation to Changes in the Character of the Diet. Results for Animals That Survived the Entire Experimental Period.

Rabbit.	Epinephrine per kilo.	Glucose in urine with			
		Mixed diet.	Basic diet.	Acid diet.	Mixed diet.
	mg.	*gm.*	*gm.*	*gm.*	*gm.*
37	0.4	1.887	0.807	1.076	1.77
38	0.4	1.807	1.764	0.724	1.045
Average..........	....	**1.847**	**1.285**	**0.900**	**1.407**
34	0.3	0.112	0.000	0.042	0.000
36	0.3	1.424	0.01	0.559	0.767
39	0.3	0.103	0.789	0.344	1.120
40	0.3	3.456	2.079	0.333	3.915
44	0.3	4.518	2.146	3.480	2.720
Average..........	....	**1.923**	**1.005**	**0.952**	**1.704**
35	0.2	0.184		0.065	0.101
29	0.2	0.398	0.023	0.278	1.015
41	0.2	0.289	0.397	0.055	0.134
45	0.2	1.697	0.124	0.815	1.400
46	0.2	0.023	0.49	0.074	0.150
Average..........	....	**0.518**	**0.258**	**0.257**	**0.660**
General average..		1.325	0.784	0.654	1.178

The Influence of Changes in the Character of the Diet upon Epinephrine Glycosuria.

The influence of the character of the diet is fairly uniform (see Tables IV and V). The output of sugar was greatest when the rabbits were given a mixed diet, and there is good agreement between the results of repeated doses upon this diet. The acid-forming diet resulted in the lowest output of sugar, averaging slightly more than one-half that upon a mixed diet. During the base-forming diet the sugar excretion was greater than with that yielding an acid ash, although the excess was, in general, slight—not more than 20 per cent.

Inasmuch as the glycosuria produced by epinephrine is dependent upon the animal's store of glycogen, these results apparently indicate that an acid-forming diet is slightly less effi-

cient in the formation of glycogen than is a base-forming diet, and that both are greatly inferior to a mixed diet in this respect. The variations in body weight seem to point in the same direction, gain in weight having been greatest and most frequent upon the mixed diet. In a comparison of the acid- and base-producing diets, gain in weight is more often seen with that yielding a basic ash.

According to Allen, the excretion of sugar after epinephrine administration is markedly influenced by diuresis. In these experiments the volume of urine was much less during the acid-forming diet of grain than with the base-producing diet of fresh vegetables, owing to the small amount of water taken by the animals while upon the grain diet. This fact may be partly responsible for the reduced output of sugar following the acid-forming diet, although an examination of the figures indicates no constant relation between the volume of urine and its sugar content.

CONCLUSIONS.

A dose of 0.3 mg. of epinephrine per kilo of body weight may be depended upon to produce glycosuria in a normal rabbit.

With a sufficient quantity of food, rabbits upon a mixed diet excrete larger amounts of sugar after epinephrine administration than when maintained upon either an acid-producing diet or one yielding a basic ash. The data given indicate that during a base-forming diet a somewhat larger output of sugar occurs subsequent to epinephrine injection than obtains under similar conditions with an acid-producing diet.

These results may possibly be interpreted to mean that there is a greater glycogen storage upon a base-producing diet than upon a dietary yielding an acid ash.

With injections of epinephrine repeated at intervals of 1 week, with rabbits there is no apparent decreased susceptibility to the drug.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVII. FURTHER EXPERIMENTS UPON THE INFLUENCE OF THE INTRAVENOUS INJECTION OF SODIUM CARBONATE UPON EPINEPHRINE HYPERGLYCEMIA AND GLYCOSURIA.

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In previous papers it has been shown that carbohydrate metabolism, in normal animals, is usually undisturbed by injections of sodium carbonate or by diets yielding an acid or basic ash. When, however, the sugar-regulating mechanism is thrown out of equilibrium, pronounced effects may result from the introduction of acid or alkali.[1]

Recently Underhill demonstrated that the hyperglycemia and glycosuria due to epinephrine are decreased by the administration of sodium carbonate. In the experiments reported in the present paper, this investigation has been extended with the object of determining whether, with the minimum effective dose of epinephrine, 0.3 mg. per kilo of body weight,[2] hyperglycemia and glycosuria can be entirely prevented by the injection of sodium carbonate.

Methods.

The experiments were performed upon healthy, full-grown rabbits. Injections into the ear vein of a solution of sodium carbonate (0.5 per cent) were followed in 30 minutes by subcutaneous administrations of 0.3 mg. of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000) per kilo of body weight. Samples of blood for the determination of sugar were

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 227.

[2] McDanell and Underhill, *J. Biol. Chem.*, 1917, xxix, 245.

taken before the administration of the carbonate, ½ hour afterward (just preceding the injection of epinephrine), and subsequently at intervals of 1 hour. The method of Lewis and Benedict was used for the estimation of blood sugar. The sugar in the urine was determined by the Allihn procedure, the copper being weighed as cupric oxide.

The data obtained may be found in Tables I and II, and in Fig. 1 the results are expressed in the form of curves. In Table I (last line) the averages for sugar of the blood and urine obtained

TABLE I.

Hyperglycemia and Glycosuria Resulting from Injections of Epinephrine. 0.3 Mg. per Kilo.

Date.	Rabbit.	Body weight.	Blood sugar content (in percentages).								Urine.	
			Normal.	Hrs. after injection of epinephrine.							Volume.	Sugar content.
				1	2	3	4	5	6	7		
		gm.									*cc.*	*gm.*
June 13	29	2,100	0.113	0.223	0.19	0.16	0.131	0.115	0.099	0.10	80	0.480
" 13	36	1,560	0.113	0.294	0.30	0.285	0.232	0.205	0.144	0.117	175	1.855
" 16	50	1,920	0.132	0.304	0.30	0.273	0.187	0.128	0.118	0.106	225	2.358
" 16	38	1,940	0.114	0.302	0.256	0.194	0.156	0.116	0.109	0.114	175	0.342
Averages........			0.118	0.281	0.261	0.228	0.176	0.141	0.117	0.109		1.259
(Underhill)........			0.13	0.36	0.430	0.46		0.37	0.32	0.19*		1.49

* After 7½ hours.

by Underhill with a dose of 1 mg. of epinephrine per kilo are included for comparison. It is evident that the hyperglycemia incident to injections of the larger dose of epinephrine is considerably greater and of longer duration than that which follows the administration of 0.3 mg. of epinephrine per kilo. Also, the maximum blood sugar content is reached sooner in the latter instance, the normal being regained in about 6 hours, or less. The average output of sugar in the urine is, however, only about 15 per cent less with the small dose of epinephrine than with injections more than three times as large.

The injection of approximately 0.5 gm. of sodium carbonate in 0.5 per cent solution ½ hour previous to the administration of epinephrine brings about a considerable diminution in the hyper-

TABLE II.

The Influence of the Intravenous Injection of Sodium Carbonate (0.5 per Cent) upon Epinephrine Hyperglycemia and Glycosuria.

Date.	Rabbit.	Body weight.	Volume of sodium carbonate solution injected.	Blood sugar content (in percentages). Normal.	Hrs. after injection of Sodium carbonate. ½	Epinephrine. 1	2	3	4	5	6	Urine. Volume.	Sugar content.
		gm.	cc.									cc.	gm.
June 19	40	2,560	120	0.10	0.112	0.323	0.237	0.153	0.127	0.112	0.095	285	0.464
" 19	39	1,540	100	0.115	0.109	0.257	0.251	0.16	0.149	0.141	0.127	200	0.524
" 22	44	2,120	125	0.111	0.131	0.297	0.350	0.344	0.295	0.289	0.228	245	2.773
" 22	49	1,620	100	0.117	0.074	0.255	0.172	0.155	0.132	0.134	0.104	250	0.147
" 26	45	2,560	125	0.111	0.093	0.167	0.168	0.128	0.117	0.108	0.093	275	0.000
" 26	53	2,640	125	0.115	0.121	0.216	0.254	0.247	0.172	0.141	0.119	220	0.000
" 28	38	1,800	110	0.106	0.096	0.224	0.217	0.185	0.146	0.129	0.108	215	0.000
" 28	50	1,800	110	0.111	0.108	0.206	0.210	0.201	0.176	0.167	0.124	230	0.156
Averages.............				0.111	0.105	0.243	0.232	0.197	0.164	0.153	0.124		0.509

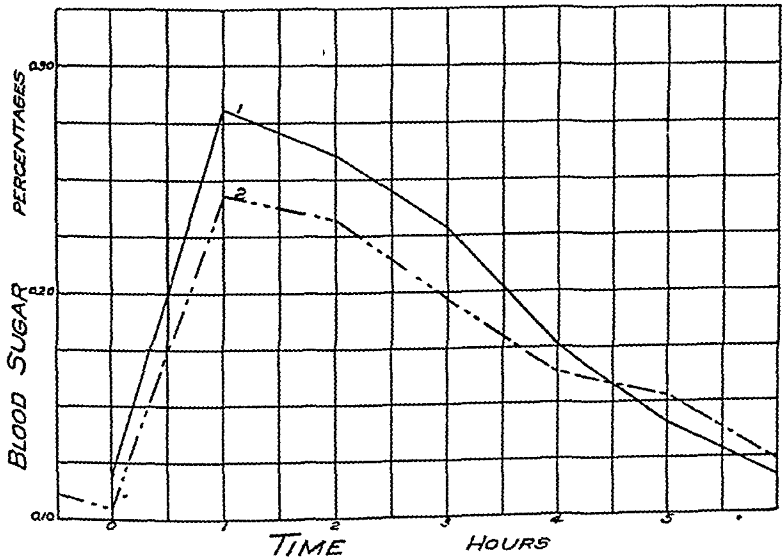

FIG. 1. Curves showing the blood sugar level after subcutaneous injections of epinephrine and the influence of the intravenous administration of sodium carbonate upon the hyperglycemia induced (average figures).

Curve 1. Epinephrine only.

Curve 2. Epinephrine and sodium carbonate.

glycemia caused by epinephrine (see Table II), although the reduction is proportionately less than that obtained by Underhill following the larger dose of epinephrine and, in some instances, larger injections of sodium carbonate. Accompanying the diminished hyperglycemia is a noticeable reduction in the output of sugar in the urine.

CONCLUSIONS.

The present experiments confirm the statement of Underhill: "The hyperglycemia and glycosuria provoked by epinephrine are both significantly decreased if sodium carbonate is administered at suitable periods of time previous to epinephrine introduction."

Intravenous injection of sodium carbonate modifies significantly the influence of the minimum effective dose of epinephrine, but does not prevent the occurrence of hyperglycemia and glycosuria. In fact, it appears that, with the smaller dose of epinephrine, hyperglycemia and glycosuria are diminished relatively less by sodium carbonate than when larger doses of epinephrine are administered.

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STUDIES IN CARBOHYDRATE METABOLISM.

XVIII. THE RELATION OF DIET TO THE GLYCOGEN CONTENT OF THE LIVER.

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It has been accepted for a long time that glycosuria accompanies partial asphyxiation. Alvaro Reynoso (see Dastre) appears to have been the first to notice this, but he made no further accurate observations on the subject. Claude Bernard confirmed this observation and found also that prolonged asphyxia caused the disappearance of glycogen from the liver. This work of Bernard was extended by Dastre, who noted that the percentage of reducing substance in the blood rose from 0.128 per cent when a dog was breathing air, to 0.253 per cent when breathing was continued for some time in a confined space. The hyperglycemia was ascribed to lack of oxygen in the blood. Araki in 1891 reported that glycosuria was caused in dogs, rabbits, and chickens by insufficient oxygen supply, although no analyses of the air breathed or of the blood gases were given.

This idea seems to have represented the predominant view of observers until Edie, as the result of experiments in which the composition of the air breathed was determined, reached the conclusion that the glycosuria occurring after partial asphyxiation is due to excess of carbon dioxide, rather than to lack of oxygen. He found that a low percentage of oxygen alone—less than 6 per cent—did not produce glycosuria, while it resulted from breathing air containing 10 to 15 per cent by volume of carbon dioxide, even if the percentage of oxygen was greater than that normally present in the atmosphere. Edie placed carbon dioxide in the class with anesthetics, all of which cause glycosuria.

This work was confirmed and extended by Edie, Moore, and Roaf. They concluded that the glycosuria attributed by Araki to low oxygen supply was actually due to accumulation of carbon dioxide. Araki's method of freeing the air from carbon dioxide—bubbling it through strong caustic potash—they found, by gas analysis, to be totally inadequate for the purpose.

In agreement with these findings of Edie and his coworkers is the conclusion of Macleod, that the property of asphyxial blood which stimulates hepatic glycogenolysis in the intact animal is the excess of carbon dioxide, and not the deficiency of oxygen which it contains.

On the other hand, that certain types of glycosuria are accompanied by a marked acapnia, rather than by an excess of carbon dioxide, was shown by Henderson and Underhill. They made analyses of the blood gases following peptone injection, piqûre, removal of the pancreas, painting of the pancreas with piperidine, laparotomy, and excessive artificial respiration. In all of these conditions they found acapnia. By supplying carbon dioxide, these investigators were able, in some instances, to prevent disturbances of the sugar-regulating mechanism.

Pavy and Bywaters, in a study of the influence of environment on enzyme action, demonstrated that with invertin (yeast) and diastase (oyster) the activity of the enzyme is increased by the addition of small quantities of acetic acid until an optimum point is reached, after which the addition of more acid leads to a diminution in the enzymic power. They showed, further, that the activity given by acid addition is susceptible of removal by a basic substance,—ammonia, sodium carbonate, and even the basic material of tap water exerting an inhibiting influence, which the subsequent addition of an acid is capable of counteracting. An environment of strong acid or alkali destroys the enzyme. With invertin, it was observed that carbon dioxide approximates acetic acid in its effect in promoting enzyme action, and with oyster diastase, the effect is similar although much reduced.

In the case of higher animals these investigators found that the enzymic power of the liver, post mortem, in which a fully activated enzyme exists, is usually not increased by addition of acid, but rather is diminished. They determined, too, that the postmortem production of sugar in the liver may be virtually checked by the injection of a 2 per cent solution of sodium carbonate into the portal system of the living animal. The addition of increasing quantities of acetic acid to the liver thus treated brings about an augmentation of enzyme activity until an optimum is reached, after which the further addition of acid leads to a decline of power. The postmortem production of acid in the liver is similar to that of sugar, of which, according to these authors, it may be the precursor. A restraining influence is exerted by a fluoride upon postmortem acid production in the liver as is done with postmortem sugar formation.

Later an attempt was made by Pavy and Godden to determine if the production of sugar by the liver, occurring in association with glycosuria, could be similarly checked, and the glycosuria thus removed, by the injection of sodium carbonate into the circulation. Chloroform glycosuria, being dependent upon the glycogen supply of the liver, was used for testing the effect of the sodium carbonate. A cat was placed under the influence of chloroform, and urine withdrawn at 15 minute intervals by means of a catheter. After glycosuria had been thoroughly established, sodium carbonate, in 3 per cent solution, to the amount of 0.75 gm. per kilo of body weight, was injected into the femoral vein. This brought about a speedy reduction in the previously existing glycosuria, which usually disappeared within 15 to 30 minutes after the completion of the injection. When normal saline or Ringer's solution was substituted for the sodium

carbonate, it exerted no influence upon the glycosuria. They found further, that, after the injection of the sodium carbonate and the consequent reduction of the glycosuria, pinching the vagus produced no effect, showing that the nerve stimulation of glycosuria was, like the chloroform glycosuria, held in check by the agency of the carbonate. In conclusion, these investigators say: "Seeing that there is evidence to show that both forms of glycosuria are due to an abnormal enzymic conversion of glycogen into sugar (attributable, there are grounds for suggesting, to acidosis development) there is nothing unconformable with what may be reasonably looked for."

Macleod and Pearce noted that after death in an etherized animal, there is usually, but not always, an acceleration in the rate of glycogenolysis.

As far back as 1882, Einhorn, working in Ehrlich's laboratory (see Ehrlich), showed that while winter frogs neither lose glycogen from their livers, nor form new glycogen, when immersed in plain sugar solution, they do lose their glycogen if the sugar solution is acidulated. But when such frogs were placed in a glucose-sodium-carbonate solution, a formation of new glycogen from available glucose, without the occurrence of glycogenolysis, was demonstrated.

In turtle livers, perfused with Ringer's solution containing hydrochloric acid, Elias observed a considerable loss of glycogen. When sodium carbonate was added to the Ringer's solution, he noted an actual glycogenesis. Elias also brought about a loss of glycogen from the livers of rabbits by the administration of 0.25 N hydrochloric acid.

As the result of a limited number of experiments, Kramer, Marker, and Murlin suggested tentatively that the glucose retained as a result of the administration of sodium carbonate to a depancreatized dog is not held back as glycogen.

While the results cited indicate that, in normal animals and in those with certain disturbances of the sugar-regulating mechanism, a large excess of alkali contributes to the formation of glycogen, and acid increases the activity of the glycogenolytic process, the influence of an acid-forming or base-forming diet upon the glycogen content of the liver seems not to have been investigated. Experiments conducted for this purpose are described in the present paper. The investigation is especially pertinent in view of the results reported in a preceding paper,[1] where there were indications that the character of the diet (its acid-base-producing properties) plays a rôle in the degree of glycosuria induced by epinephrine administration.

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 245.

Series I.

Methods.—A subcutaneous injection of epinephrine (Parke, Davis and Company, adrenalin chloride, 1: 1,000), 1 mg. per kilo of body weight, was given to each of ten full-grown rabbits for the purpose of freeing their livers of the excess of glycogen. The rabbits, in two groups of five each, were placed in pens. To those of Group A was given a base-producing diet of carrots, about a pound daily for each rabbit. Group B received an ample acid-producing diet of oats and cracked corn. Water was always available. One rabbit of Group B died 4 days later and was not replaced. The animals were kept upon the designated rations

TABLE I.

The Influence of Diet upon the Glycogen Content of the Liver.
Series I, Group A, July 11–17, Carrot Diet.

Rabbit.	29	34	36	38	40	Average.
Weight						
July 11, gm.	2,240	1,700	1,540	1,720	2,520	
" 17, "	2,200	1,700	1,440	1,720	2,500	
Weight of liver, gm.	52	53	42	49	82	
Glycogen.						
Glucose × 0.927, gm.	2.83	3.65	1.86	2.08	3.12	2.71
Per cent of weight of liver	5.44	6.91	4.43	4.24	3.81	4.87
Per kilo of body weight, gm.	1.28	2.15	1.29	1.21	1.25	1.42

for 6 days, then killed, and the glycogen in the livers was estimated according to Pflüger's method.[2] After hydrolysis the glucose was determined by the Allihn procedure, the copper being weighed as cupric oxide.

From Table I it may be seen that, of the rabbits in Group A upon the carrot diet, two maintained their weight, while one lost 20, another 40, and another 100 gm. However, the percentage of glycogen in the livers and the amount of glycogen per kilo of body weight have no direct relation to these slight changes in weight. It is evident that the variation in amount of

[2] Pflüger, E., *Arch. ges. Physiol.*, 1906, cxiv, 242; *Abderhalden's Handb. biochem. Arbeitsmethoden*, 1910, ii, 162.

glycogen per kilo of body weight, with the exception of Rabbit 34, is slight.

Table II shows that the rabbits of Group B, upon an unlimited grain diet, all lost weight, from 20 to 300 gm.; and that, with the exception of Rabbit 53, the percentage of glycogen in the livers, as well as the amount per kilo of body weight, was less than that of the rabbits of Group A, upon the carrot diet. However, the fact that Rabbit 53 stored a much greater proportion of glycogen than did any of the rabbits of Group A indicated that there were undoubtedly other factors than the character of the diet to be considered. Since this rabbit had practically maintained its weight, while the others had lost considerably, it seemed possible that they had failed to store glycogen because of an insufficient intake of food, as it is our experience that many rabbits do not relish a diet of grain only.

TABLE II.

The Influence of Diet upon the Glycogen Content of the Liver.
Series I, Group B, July 11–17, Grain Diet.

Rabbit	45	50	51	53	Average
Weight.					
July 11, gm.	2,480	1,740	1,860	2,500	
" 17, " .	2,300	1,440	1,780	2,480	
Weight of liver, gm	60	46	47	70 5	
Glycogen.					
Glucose × 0.927, gm.	0 697	1 055	1 693	6 956	2 6
Per cent of weight of liver	1 16	2 29	3 6	9 87	4 65
Per kilo of body weight, gm.	0 303	0 733	0 951	2 805	1 3

While these results apparently indicated a better storage of glycogen upon a base-forming than upon an acid-forming diet, further experiments were planned in which the amount of food consumed by each rabbit should be known.

Series II.

Methods.—The rabbits of Series II were kept in separate metabolism cages for 1 week. After the preliminary injection of epinephrine, those of Group A were given 500 gm. of carrots a day;

those of Group B, 100 gm. of oats (instead of corn and oats). One rabbit of Group A died on the 3rd day. The food value of the diets was estimated from the percentage composition of carrots given by Sherman;[3] and of oats, by Henry and Morrison.[4]

TABLE III.

The Influence of Diet upon the Glycogen Content of the Liver.
Series II, Group A, October 23–30, Carrot Diet.

Rabbit.	73	76	77	Average.
Weight.				
October 23, gm.	2,520	1,920	1,900	
" 30, "	2,740	1,920	1,920	
Food eaten.				
October 24, gm.	98	500	480	
" 25, "	327	500	467	
" 26, "	500	500	380	
" 27, "	500	500	500	
" 28, "	500	500	500	
" 29, "	500	500	500	
" 30, "	470	500	366	
Total, gm.	2,895	3,500	3,193	
Carbohydrate, gm.	214	259	236	
Per kilo, gm.	78.09	134.9	122.9	111.96
Calories	1,038	1,256	1,145	
Per kilo	378.7	654.2	596.4	543.1
Weight of liver, gm.	81.8	70.7	69.8	
Glycogen.				
Glucose × 0.927, gm.	7.1	5.16	4.94	5.73
Per cent of weight of liver	8.67	7.3	7.08	7.68
Per kilo of body weight, gm.	2.59	2.69	2.57	2.62
Per 100 gm. carbohydrate, gm.	3.24	1.99	2.09	2.44
Per 1,000 calories, gm.	6.84	4.11	4.31	5.09

Table III shows that in Series II none of the rabbits of Group A upon the carrot diet lost weight, one of them (Rabbit 73) gaining 220 gm.; and that they all stored more glycogen, absolutely and relatively, than the carrot-fed rabbits of Series I (see Table I).

[3] Sherman, H. C., The Chemistry of Food and Nutrition, New York, 1911, 323.

[4] Henry, W. A., and Morrison, F. B., Feeds and Feeding, New York, 1915, 634.

That great variation was manifest in Group B of Series II, as of Series I, is indicated in Table IV. Two of the rabbits gained weight (Rabbits 68 and 71, 20 and 100 gm. respectively), while

TABLE IV.

The Influence of Diet upon the Glycogen Content of the Liver.
Series II, Group B, October 23–30, Oat Diet.

Rabbit.	68	69	70	71	72	75	Average.
Weight.							
October 23, gm..	2,360	2,520	2,760	1,720	2,580	2,600	
" 30, " ..	2,380	2,460	2,560	1,820	2,500	2,440	
Food eaten.							
October 24, gm..	29	10	39	36	25	7	
" 25, " ..	85	22	17	83	94	70	
" 26, " ..	95	42	65	90	98	75	
" 27, " ..	95	68	91	78	98	93	
" 28, " ..	50	66	72	43	98	90	
" 29, " ..	95	65	80	100	100	50	
" 30, " ..	88	67	49	65	92	19	
Total, gm.....	537	340	413	495	605	404	
Carbohydrate, gm........	320	203	246	295	361	241	
Per kilo, gm.	134.5	82.52	96.1	162.1	144.4	98.77	119.73
Calories.......	1,805	1,144	1,318	1,664	2,035	1,359	
Per kilo.....	758.4	465	514.8	914.3	814.1	556.9	670.6
Weight of liver, gm..............	79.5	67.5	75.6	65.1	102.9	68.4	
Glycogen.							
Glucose × 0.927, gm...........	8.68	4.43	3.05	5.97	11.61	0.92	5.78
Per cent of weight of liver..........	10.91	6.56	4.04	9.18	11.29	1.34	7.22
Per kilo of body weight, gm....	3.65	1.8	1.19	3.28	4.64	0.38	2.49
Per 100 gm. carbohydrate, gm.	2.71	2.18	1.24	2.03	3.22	0.38	1.96
Per 1,000 calories, gm......	4.81	3.87	2.31	3.59	5.7	0.68	3.49

the other four lost, the amount varying from 60 to 200 gm. Rabbits 68 and 72 stored more glycogen, and Rabbits 68, 71, and 72 each showed a greater percentage of glycogen in the liver, as well as a

larger amount of glycogen per kilo of body weight, than did any of the carrot-fed rabbits. Much less glycogen was stored by the other three rabbits of this group than by those of Group A. When averages are considered, it may be seen that the proportion of glycogen in the liver and the amount per kilo of body weight were slightly greater with the carrot-fed than with the oat-fed animals, irrespective of the food intake.

The importance of the quantity of food ingested is, however, demonstrated by the results in Series II. Rabbits 68, 71, and 72 (Table IV), which stored the greatest proportion of glycogen, received by far the largest number of calories per kilo. Rabbit 75, which stored the least glycogen, ate less during the last 2 days than any of the others, although its total food intake was not the lowest.

It is equally evident from a study of Tables III and IV that the quantity of food consumed is not the sole determinant of the glycogen storage. Of the six rabbits in Group B, oat-fed (Table IV), four (Rabbits 69, 70, 71, 75) stored less glycogen per 1,000 calories of food eaten than any of the rabbits of Group A, carrot-fed (Table III), only one of Group B (Rabbit 72) manifesting greater ability in this respect than the average of Group A. This oat-fed rabbit (No. 72), which stored 11.6 gm. of glycogen, received more than twice as many calories per kilo as Rabbit 73, carrot-fed, which stored 7.1 gm. of glycogen. In fact, the 7.1 gm. of glycogen were stored by Rabbit 73, upon a base-forming diet, with an intake of 378.7 calories per kilo. The lowest fuel value of the diet of any of the oat-fed rabbits was 465 calories per kilo (Rabbit 69). Although the average fuel value of the acid-forming diet of the rabbits of Group B (670.6 calories) was more than 20 per cent greater than that of the base-forming diet of Group A (543.1 calories), the average amount of glycogen stored per 1,000 calories was 30 per cent less in the former instance than in the latter. With respect to the efficiency of the carbohydrate in the two diets, the differences, although less, point in the same direction. With an average intake of carbohydrate per kilo slightly greater, the rabbits upon an acid-forming diet (Group B) stored about 20 per cent less glycogen per 100 gm. of carbohydrate received than those upon the base-producing diet (Group A).

CONCLUSIONS.

The results obtained indicate that in normal rabbits a base-forming diet is somewhat more efficient in the formation of glycogen than an acid-producing diet. This is in agreement with the results of previous investigators, who have found that an excess of alkali contributes to the accumulation of glycogen. Notwithstanding this fact, it has also been demonstrated that a large storage of glycogen may take place upon an acid-forming diet when sufficient food is ingested.

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STUDIES IN CARBOHYDRATE METABOLISM.

XIX. THE INFLUENCE OF THE INTRAVENOUS INJECTION OF SODIUM CARBONATE UPON THE HYPERGLYCEMIA AND GLYCOSURIA FOLLOWING THE SUBCUTANEOUS ADMINISTRATION OF GLUCOSE.

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In the normal organism of the rabbit, the blood-sugar-regulating mechanism is usually undisturbed by injections of alkali or by the ingestion of an acid- or base-producing diet. On the other hand, there is evidence that the glycogenetic function of the body is more favorably influenced by a base-forming diet than it is by a dietary yielding an acid ash.[1] Under conditions where the sugar-regulating mechanism is thrown out of equilibrium, as by epinephrine administration, the introduction of alkali exerts a distinct influence upon carbohydrate metabolism, as evidenced by the diminished output of sugar in the urine. In general, it would appear that alkali has a distinct tendency to conserve the carbohydrate of the body, and in this action the glycogenetic and glycogenolytic processes are probably actively concerned.

If the alkali shows a distinctly favorable influence upon glycogen storage, it would appear reasonable to assume that the simultaneous introduction of alkali and glucose should yield less urinary sugar than glucose alone. The present communication gives the results of an endeavor to determine this question, the problem being investigated with respect to the content of blood sugar, and the urinary sugar excretion.

[1] McDanell, L., and Underhill, F. P., *J. Biol. Chem.*, 1917, xxix, 255.

Methods.

Healthy, full-grown rabbits, upon a mixed diet, were employed for these experiments. During the first part of the work, Merck's purified dextrose was used, with the exception of three injections of commercial glucose. Afterward Kahlbaum's pure dextrose was administered. Kahlbaum's anhydrous sodium carbonate and sodium chloride were employed. After a sample of blood was obtained for the determination of the normal sugar content, the rabbit received a subcutaneous injection of glucose, about 5 gm. per kilo of body weight, in 30 per cent solution. The blood sugar content was determined by the Lewis-Benedict method at the end of ½ hour and 1 hour, and each subsequent hour until the 5th. When sodium carbonate was given, an infusion of approximately 0.5 gm., in 0.5 per cent solution, was made into the ear vein 15 minutes after the glucose injection. In substituting sodium chloride for the carbonate, a 0.364 per cent solution was employed, that being isotonic with the 0.5 per cent sodium carbonate.[2] To some animals three or four injections were given. Sugar was injected at intervals of not less than 1 week. The animals were kept in metabolism cages and the urine was collected for 24 hours after each injection. Urinary sugar was estimated according to the Allihn procedure.

The Hyperglycemia and Glycosuria Following the Subcutaneous Administration of Glucose.

According to Allen, the rabbit possesses a greater assimilation limit for glucose than the dog. This assertion is based upon the work of various investigators, particularly Heilner and Fichtenmayer. A perusal of the literature emphasizes, however, that there is a diversity of opinion concerning the assimilation limit of the rabbit for subcutaneously injected glucose. Thus, Frugoni and Stradiotti place the threshold dose for the rabbit at 2.35 gm. per kilo, as compared with 3 gm. per kilo for the guinea pig. Allen designates these results atypical. Süssenguth injected 5 to 10 gm. of glucose per kilo, and observed glycosuria with the

[2] Jones, H. C., *Z. physik. Chem.*, 1893, xi, 113; 1893, xii, 636. Abegg, R., Handb. anorgan. Chem., Leipsic, 1905, ii, pt. i, 228, 302.

larger doses. Marrassini reported glycosuria usually after doses of approximately 10 gm. of glucose per kilo. Heilner, with doses of 12 to 13 gm. of glucose per kilo, observed slight glycosuria in some instances, and none in others. One rabbit showed neither glucose nor albumin in the urine after a subcutaneous dose of 49.8 gm. of glucose. Fichtenmayer reported no glycosuria in rabbits, some of them fasting, with injections of glucose in amounts varying from 10 to 23 gm. per kilo. Allen observed

TABLE I

The Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of a 30 per Cent Solution of Glucose.

First Injections.

Date	Rabbit	Weight	Volume of glucose solution injected	Blood sugar content (in percentages)							Urine.	
				Normal	Hrs after injection of glucose						Volume	Sugar content
					½	1	2	3	4	5		
		gm	*cc*								*cc*	*gm*
July 11.	54	2,620	44*	0 134	0 273	0 315	0 287	0 287	0 21	0 18	70	0 86
" 11..	55	3,040	50.5*	0 107	0 215	0 205	0 169	0 186	0 153	0 155	43	0 39
" 13..	57	2,460	41*	0 105	0 226	0 239	0 258	0 226	0 183	0 145	120	0 516
" 13.	58	2,720	44*	0 ·116	0 217	0 235	0 238	0 20	0 175	0 171	76	0 481
" 18.	59	2,460	41*	0 091	0 256	0 282	0 271	0 229	0 177	0 146	45	0 317
" 18.	60	1,880	31 3*	0 13	0 227	0 237	0 188	0 157	0 137	0 138	23	0 271
Sept. 29..	61	2,960	44 4†	0 12	0 325	0 343	0 24	0 179	0 155	0 145	75	0 297
" 29.	62	2,260	34†	0 126	0 274	0 32	0 287	0 255	0 207	0 199	65	0 248
Oct. 2.	63	1,880	28†	0 131	0 268	0 246	0 202	0 189	0 148	0 142	49	0 035
" 2.	64	2,060	32 5†	0 12	0 238	0 24	0 173	0 114	0 131	0 136	46	0.037
" 6.	67	1,840	27 6†	0 114	0 202	0 20	0 17	0 156	0 15	0 137	28	0 081
Average..				**0 1176**	**0 247**	**0 262**	**0 226**	**0 198**	**0 166**	**0 154**		**0 321**

* Dextrose, Merck.
† Dextrose, Kahlbaum.

glycosuria in one rabbit with a dose of 5 gm. per kilo, while others failed to develop glycosuria with doses as large as 7.7 gm. per kilo.

It is apparent from these diverse results that renewed investigation was essential for the determination of the assimilation limit for subcutaneously introduced glucose in the rabbit. It was also important to discover the influence upon the assimilation limit of repeated injections of sugar.

Our experiments have been conducted with doses of glucose approximating 5 gm. per kilo of body weight. The results may be seen in Tables I and II. Table I includes first injections, and Table II, second, third, and fourth injections. While wide variations are manifest in the assimilation limit of different animals, and of the same animal at different times, in only one instance did a dose of 4 to 5 gm. of glucose per kilo fail to produce

TABLE II.

The Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of a 30 per Cent Solution of Glucose.

Second, Third, and Fourth Injections.

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Blood sugar content (in percentages).							Urine.	
					Normal.	Hrs. after injection of glucose						Volume.	Sugar content.
						½	1	2	3	4	5		
		gm.		*cc.*								*cc.*	*gm.*
July 31...	55	2,520	4	50.5*	0.13	0.361	0.408	0.408	0.392	0.378	0.352	63	4.334
" 31...	57	2,180	4	41*	0.128	0.317	0.353	0.37	0.357	0.275	0.208	65	0.796
. 20...	61	2,340	4	44.4†	0.102	0.231	0.24	0.192	0.178	0.181	0.216		
9...	64	2,020	2	32.5†	0.148	0.391	0.405	0.312	0.221	0.156	0.164	45	0.296
16...	64	1,840	3	32.5†	0.119	0.244	0.28	0.238	0.266	0.272	0.28		
" 11...	65	1,900	2	27†	0.124	0.215	0.204	0.141	0.15	0.13	0.122	72	0.180
" 25...	65	1,900	4	27†	0.125	0.198	0.227	0.20	0.15	0.119	0.112	110	0.000
" 11...	66	1,640	2	25.2†	0.14	0.323	0.346	0.353	0.255	0.156	0.134	54	0.316
" 25...	66	1,760	4	25.2†	0.144	0.281	0.292	0.304	0.26	0.160	0.122	146	0.147
" 13...	67	1,740	2	27.6†	0.139	0.21	0.189	0.156	0.159	0.15	0.16	35	0.035
" 20...	67	1,860	3	27.6†	0.126	0.245	0.24	0.199	0.149	0.138	0.134	84	0.115
Average..........................					0.129	0.274	0.289	0.261	0.231	0.192	0.182		0.691

* Commercial glucose.

† Dextrose, Kahlbaum.

glycosuria (Rabbit 65, fourth injection, Table II). As indicated by the glycemia, the tolerance of this animal was above the average. According to Underhill and Closson, Underhill and Hilditch, and Allen, 5 gm. of glucose per kilo never produce glycosuria (barring accidental traces) in any normal dog. Hence the results here obtained assign to the rabbit a lower assimilation limit for subcutaneously administered glucose than is ordinarily accepted for the dog.

In general, after subcutaneous administration of glucose, the blood sugar level rises rapidly during the first half hour, and more slowly during the second half hour. A maximum is reached 1 hour after the administration of glucose, and thereafter is a gradual decline to normal.

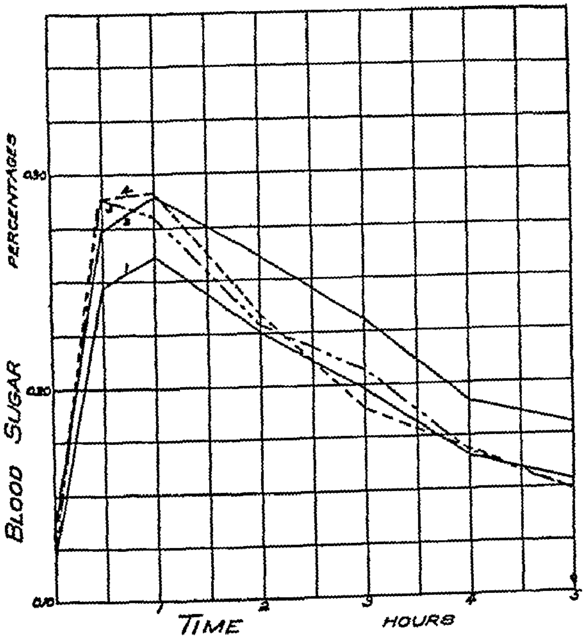

Fig. 1. Curves showing the blood sugar level after subcutaneous injections of glucose, and the influence of the intravenous administration of sodium carbonate and sodium chloride upon the hyperglycemia induced (average figures).

Curve 1. Glucose only (first injections).
" 2. " " (second, third, and fourth injections).
" 3. " and sodium carbonate.
" 4. " " " chloride.

Allen found no diminution in the power of cats and dogs to utilize subcutaneously injected glucose, as the result of repeated injections. In our experiments with rabbits, the average maximum hyperglycemia incident to first injections is just a little lower than from subsequent injections, and the average output of sugar in the urine from first injections is somewhat smaller than that obtained from the later injections. In Fig. 1 may be

seen the striking parallelism between the average curve of blood sugar content obtained from the initial sugar injection (Curve 1), and that yielded by repeated sugar administration (Curve 2). The data presented indicate that with rabbits, in general, repetition of subcutaneous injections of glucose, under the experimental conditions, is also without significant influence upon the assimilation limit of sugar.

The Influence of the Intravenous Injection of Sodium Carbonate upon the Hyperglycemia and Glycosuria Following the Subcutaneous Administration of Glucose.

Before drawing conclusions concerning the influence of sodium carbonate upon the assimilation limit of glucose, it was deemed desirable to ascertain the effect of some other solution with the same osmotic pressure, but without the presence of alkali. For this purpose the 0.364 per cent solution of sodium chloride was employed. It is evident from Table IV and Fig. 1 (Curve 4) that the sodium chloride solution is without appreciable influence upon the assimilation limit of glucose in the rabbit, under the experimental conditions selected.

When glucose is administered subcutaneously and sodium carbonate is injected into the blood stream shortly afterward, the resulting hyperglycemia shows marked variations with different animals (see Table III). If, however, an average of these figures is taken, the resulting curve which may be formed (Fig. 1, Curve 3) is similar to that yielded by the average figures for the injection of glucose alone (Curves 1 and 2, Fig. 1). The maximum is almost identical with that for repeated injections of glucose only, but is reached in ½ hour rather than in 1 hour. The administration of sodium carbonate has no significant influence upon the glycosuria induced by the subcutaneous injection of glucose.

CONCLUSIONS.

The tolerance of the rabbit for subcutaneously injected glucose is somewhat below the value generally accepted for the dog.

When glucose is repeatedly administered, at intervals of 1 week, the assimilation limit of rabbits is not influenced appreciably.

TABLE III.

The Influence of Sodium Carbonate (0.5 per Cent Solution) upon the Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of Glucose (30 per Cent Solution).

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Volume of sodium carbonate injected.	Blood sugar content (in percentages). Normal.	Hrs. after injection of glucose. ½	1	2	3	4	5	Urine. Volume.	Sugar content.
		gm.		cc.	cc.								cc.	gm.
July 20	54	2,700	2	44*	130	0.106	0.22	0.215	0.206	0.189	0.194	0.147	70	0.272
" 20	55	3,080	2	50.5*	130	0.104	0.188	0.161	0.141	0.159	0.173	0.169	100	0.462
" 21	57	2,320	2	41*	120	0.115	0.281	0.272	0.259	0.261	0.23	0.204	100	0.7
" 28	59	2,460	3	41*	125	0.118	0.335	0.328	0.286	0.26	0.174	0.146	175	0.567
Oct. 4	65	1,800	1	27†	100	0.115	0.253	0.254	0.21	0.195	0.156	0.138	96	0.068
" 4	66	1,680	1	25.2†	100	0.135	0.274	0.266	0.209	0.185	0.149	0.139	84	0.000
" 6	61	2,840	2	44.4†	130	0.122	0.428	0.423	0.281	0.24	0.142	0.132	94	0.985
" 9	63	1,760	2	28.2†	100	0.129	0.324	0.318	0.242	0.164	0.123	0.12	80	0.083
Average						0.118	0.288	0.28	0.229	0.207	0.168	0.149		0.392

* Dextrose, Merck.
† Dextrose, Kahlbaum.

TABLE IV.

The Influence of Sodium Chloride (0.364 per Cent Solution) upon the Hyperglycemia and Glycosuria Resulting from the Subcutaneous Injection of Glucose (30 per Cent Solution).

Date.	Rabbit.	Weight.	No. of injection.	Volume of glucose solution injected.	Volume of sodium chloride injected.	Blood sugar content (in percentages). Normal.	Hrs. after injection of glucose. ½	1	2	3	4	5	Urine. Volume.	Sugar content.
		gm.		cc.	cc.								cc.	gm.
July 27	55	2,960	3	50.5*	130	0.112	0.353	0.34	0.209	0.181	0.18	0.147	170	0.433
" 27	57	2,280	3	41*	120	0.111	0.343	0.402	0.398	0.296	0.219	0.18	140	0.475
Aug. 1	59	2,360	4	41†	125	0.119	0.311	0.325	0.293	0.224	0.163	0.145	170	0.935
Oct. 13	61	2,580	3	44.4‡	130	0.125	0.306	0.288	0.179	0.147	0.153	0.137	235	0.245
" 18	65	1,860	3	27‡	100	0.118	0.204	0.159	0.14	0.122	0.127	0.125	220	0.000
" 18	66	1,740	3	25.2‡	100	0.139	0.212	0.231	0.183	0.167	0.169	0.16	205	0.000
Average						0.121	0.288	0.291	0.234	0.189	0.169	0.149		0.348

* Dextrose, Merck.
† Commercial glucose.
‡ Dextrose, Kahlbaum.

The influence of the intravenous injection of sodium carbonate upon the hyperglycemia and glycosuria in normal rabbits, following the subcutaneous administration of glucose is not significant. Wide variations in the response of different animals are exhibited. The results are in agreement with those of a previous paper,[3] in which the data indicated that the sugar-regulating mechanism of a normal animal is not usually influenced by the intravenous injection of small quantities of sodium carbonate.

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STUDIES IN CARBOHYDRATE METABOLISM.

XX. NEW EXPERIMENTS UPON THE MECHANISM OF SALT GLYCOSURIA.

By LOUISE McDANELL and FRANK P. UNDERHILL.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.

(Received for publication, January 18, 1917.)

It was noted by Bock and Hoffman in 1871 that large amounts of a 1 per cent solution of sodium chloride, injected into the arterial circulation of rabbits, gives rise to diuresis, followed by glycosuria. Külz demonstrated that similar effects follow the introduction of other sodium salts—the acetate, carbonate, valerianate, and succinate—as well as the chloride. Martin Fischer confirmed these results. Moreover, he showed that, when glycosuria has been established, it may be arrested by subsequent injections of a mixture of the sodium salt and calcium chloride. A reappearance of sugar in the urine is brought about by the replacement of the calcium solution by that of a sodium salt. Fischer also found that the rapidity with which sugar appears in the urine varies directly with the concentration of the salt solution employed, that the degree of diuresis bears no definite relation to the quantity of sugar excreted, and that albuminuria is a frequent accompaniment of this glycosuria. Results similar to those of Fischer were obtained by Brown. Owing to the fact that the latter investigator used an anesthetic which of itself is capable of producing glycosuria, Fischer discounts the value of Brown's results. Later, Fischer (1905) observed that when sodium salts are injected directly into the arterial system (into the axillary artery, hence through the vertebral arteries to the spinal bulb) glycosuria occurs sooner than after injections into the venous circulation. From these facts he concluded that, in the glycosuria provoked by the introduction of sodium salts into the circulation, two factors are involved: one, an action upon the kidney by which diuresis is induced; the second, an influence upon the diabetic center in the spinal bulb, which causes glycosuria.

Underhill and Closson demonstrated a difference between the glycosuria following the introduction of solutions of sodium chloride into the venous and into the arterial circulation of the rabbit. They attribute the glycosuria in the former instance, which is accompanied by polyuria, to an increased permeability of the kidney. This permeability may be decreased by the injection of a mixture of sodium chloride and calcium chloride, as

indicated by the temporarily decreased flow of urine and diminished or inhibited excretion of sugar. Also, they found this form of glycosuria to be accompanied by a hypoglycemia, which gave place to normal blood sugar, or even hyperglycemia, when the excretion of sugar in the urine is inhibited by the injection of a calcium salt. On the other hand, injection of sodium chloride into the cerebral arterial circulation induces glycosuria with no polyuria, but with an accompanying hyperglycemia. They believe that, in the latter instance, the increased content of sugar in the blood may be referred to disturbances of respiratory processes, dyspnea, provoked by the introduction of sodium chloride.

Further work upon this subject led McGuigan and Brooks to state: "The permeability of the kidney is of small import in the mechanism of experimental glycosuria."

Underhill and Kleiner, considering this conclusion not justified by the data given, repeated certain of the experiments of McGuigan and Brooks, and as the result, say: "Renewed investigation has afforded no occasion to modify the conclusions already reached regarding the mechanism of salt glycosuria. Under appropriate conditions in the rabbit glycosuria due to renal permeability induced by sodium chloride injections can be inhibited by injections of calcium chloride. If calcium chloride is appropriately introduced glycosuria fails to be evinced even when free sugar is injected in addition."

More recently Hirsch failed to obtain glycosuria, without an accompanying hyperglycemia, after intravenous injections of sodium chloride. Since his results are diametrically opposed to those obtained by Underhill and his coworkers, the subject of the mechanism of salt glycosuria has been submitted to renewed investigation. Particular interest attaches to the question whether at the period of glycosuria provoked by salt infusion there is augmentation of the level of blood sugar. It is evident that, if blood sugar content is not increased under these circumstances, as asserted by Underhill and his coworkers, a change in renal permeability must be assumed. Otherwise, in the absence of hyperglycemia, there is no obvious explanation for the existing glycosuria.

Methods.

These experiments were performed upon full-grown, well fed rabbits, without anesthesia. The "bootjack" rabbit holder used in this laboratory has been described elsewhere.[1] The blood sugar was determined by the Lewis-Benedict method. Haldane's procedure for the estimation of hemoglobin[2] was followed. Benedict's qualitative reagent was used in testing for

[1] McDanell, L., *J. Lab. and Clin. Med.*, 1916, i, 804.
[2] Haldane, J., *J. Physiol.*, 1901. xxvi, 497.

sugar in the urine. The solution of sodium chloride was injected continuously, under pressure, into the marginal vein of an ear. Samples of blood for the determination of blood sugar and hemoglobin were obtained from a vein of the other ear. The bladder was emptied at intervals by pressure through the abdominal wall.

The results of seven experiments are given in the accompanying table. From these data it may be seen that *the intravenous injection of M/2 sodium chloride invariably induced glycosuria within a period of 15 to 20 minutes.* The volume of solution required to provoke the appearance of sugar in the urine varied from 130 cc. in Experiment 4 to 224 cc. in Experiment 3, the average of the six experiments being 176 cc.

The glycosuria observed was not due to hyperglycemia, for, although the hypoglycemia noted by Underhill and Closson was not in evidence, *the highest blood sugar content exhibited at the end of an injection was 0.14 per cent* (Experiment 6). Under these circumstances, *sugar in the urine cannot be due to a condition of hyperglycemia.* It is therefore apparent that the glycosuria induced must be ascribed to a renal factor, as originally proposed by Underhill and Closson.

In two instances (Experiments 2 and 3) a hyperglycemia was found 1 hour after the end of the injection. Rabbit 79 (Experiment 2) was prostrated as the result of the injection, but recovered rapidly. In Rabbit 80 (Experiment 3) which developed a hyperglycemia of 0.286 per cent 1 hour after the injection, there was much greater prostration, in fact a paralysis, from which the animal did not recover. The prostration appears to have been due to the poisonous effects of a large excess of the sodium salt. Wherever such a condition was encountered in our experiments, hyperglycemia accompanied it. In this connection, it is to be noted that Rabbit 80 excreted a relatively small amount of urine, and also that the fall in the percentage of hemoglobin was greater than with any of the other rabbits.

The figures for blood sugar cannot be accounted for by mere dilution of the blood as a result of the salt solution administered. This may be seen from the values for the hemoglobin, determined before and just after the injection.

TABLE.

Time.	Solution injected. (In last interval of time.)	Urine excreted. (In last interval of time.)	Hemoglobin.	Blood sugar.	Remarks.
Experiment 1. Rabbit 78, 2,700 gm. October 24, injection of M/6 sodium chloride.					
	cc.		*per cent*	*per cent*	
10.00			55	0.115	
10.10					Injection begun.
10.25	100				No sugar.
10.40	94				" "
10.55	114				" "
11.10	71				" "
11.25	102				" "
11.40	77				" "
11.55	112				" "
12.10	83				" "
12.25	100				" "
12.40	82				" "
12.55	128				" "
1.10	85				" "
1.25	129				Sugar.
1.35	44				"
	1,321				Injection stopped.
1.45			50	0.138	
3.00			56	0.116	Rabbit had poor use of muscles when first taken from board. Recovered rapidly.
Experiment 2. Rabbit 79, 2,240 gm. October 26, injection of M/2 sodium chloride.					
9.40			55	0.119	
9.47					Injection begun.
9.53	60				No sugar.
9.57	102	55			Sugar.
10.02	34	45			"
10.07	21	30			"
10.12	23	31			"
10.17	22	31			"
10.22	31	32			

TABLE—*Continued.*

Time.	Solution injected. In last interval of time.	Urine excreted. In last interval of time.	Hemoglobin.	Blood sugar.	Remarks
Experiment 3. Rabbit 80, 2,360 gm. October 27, injection of M/2 sodium chloride.					
	cc.		*per cent*	*per cent*	
9.45			42	0.119	
9.53					Injection begun.
9.58	60				
10.03	54	20			No sugar.
10.08	54	16			Trace of sugar.
10.13	56	14			Sugar.
10.18	44	8			"
10.23	31	6			"
	299	64			Injection stopped.
10.25			32	0.116	
11.25			43	0.286	Rabbit paralyzed. Dead next morning.
Experiment 4. Rabbit 81, 2,580 gm. October 28, injection of M/2 sodium chloride.					
10.00			58	0.111	
10.08					Injection begun.
10.13	58	16			No sugar.
10.18	34	19			" "
10.23	38	45			Sugar.
10.28	37	70			"
	167	150			Injection stopped.
10.30			56	0.121	
11.30			58	0.136	Rabbit in excellent condition at end of experiment.

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STUDIES OF AUTOLYSIS.

V. THE INFLUENCE OF BILE ON AUTOLYSIS.

BY H. C. BRADLEY AND JOSEPH TAYLOR.

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(Received for publication, January 15, 1917.)

In a recent article[1] Tatum describes the marked disintegration of tissues immersed in bile or its salts. In the peripheral zone, where there was evident penetration by the bile, cytolysis was apparent within 4 hours or less, the nuclear material and cytoplasm showed alterations in staining reactions, and in some cases disintegration was so complete as to leave only connective tissue structures. The facts developed in this study are of importance physiologically and pathologically. They suggest in a striking way the possibilities of tissue damage which may result from contact with bile. At the same time the mechanism by which this cytolysis is induced does not appear to us to have been conclusively established by the author. The conclusion reached was that this disintegration of tissue cells by bile was correlated with autolysis. "*This cytolysis is produced by virtue of the co-enzyme or activating action of the constituents of bile on the autolytic enzymes or processes.*" It should be pointed out that this conclusion is chemical, while the observations leading to it were wholly histological. Furthermore, if the conclusion is valid it establishes an important chemical mechanism connected with the autolytic process about which there still exists some doubt and for which no direct evidence has yet been found; *i.e.*, activation of the enzymes.

In the data presented by Tatum the following points appear to us of significance. First, the reaction described is one of tremendous speed compared with normal autolysis. A block of tissue

[1] Tatum, A. L., *J. Biol. Chem.*, 1916, xxvii, 243.

disintegrates twenty times as fast in bile as it does in dilute acid. Yet dilute acid is by far the best autolytic accelerator hitherto found. If the action of bile is one of enzyme activation it must produce an amount of active enzyme many times that found in normal tissue or in tissue of optimal acidity. Such an enormous alteration of activity leads us to question whether the explanation given is sound. In the second place, the fact that fibrin and heat-coagulated tissues were not dissolved in bile while the fresh tissues were does not necessarily imply that enzymes are required in the process. The solvent action of the bile salts on the proteins themselves has not been ruled out. Third, the order of rapidity of disintegration follows to a striking degree the order of rapidity of autolysis. The glands like liver and spleen go to pieces rapidly, while muscle tissue disintegrates slowly. There may indeed be some fundamental connection between the disintegration of these tissues and the ease with which their proteins hydrolyze without involving any further connection between the action of bile and the autolytic enzymes. Finally, there is a certain lack of distinction between the terms cytolysis and autolysis as used by the author, which has, we believe, led to the errors of interpretation. Autolysis was the term given by Jacoby[2] to the process of tissue disintegration involving deep-seated hydrolysis of the proteins. It was so used by Hedin, Rowland, Preti, and a score of others following Jacoby's work. It has carried the same implications up to the present time, with occasional broadening of the term to include other hydrolyses going on in postmortem tissue, such as the digestion of fats or glycogen. Where not otherwise specified the term autolysis signifies tissue autoproteolysis. On the other hand, cytolysis is a morphological term; it may or may not involve deep-seated chemical change back of the cell disintegration. It is used to describe the laking of the red corpuscles by water or other hemolytic agents, it is also used to describe the cell disintegration of a tissue after death. The one is probably a physical phenomenon, the other is certainly chemical. In certain cases cytolysis and autolysis are synonymous, in others they are not.

The results of Tatum's investigations do not constitute a proof of the activation of an enzyme, since no test of enzyme activity

[2] Jacoby, M., *Z. physiol. Chem.*, 1900, xxx, 174.

was made. The data are subject to interpretation in a variety of other ways, and indeed it may be questioned whether autolysis is involved at all in the process described. The microscopical picture of cytolysis is inadequate to demonstrate either autolysis or enzyme activation. For this reason we have subjected the problem to a definite chemical test, using the technique described in previous papers,[3] and estimating the speed and extent of autolysis by the amino-acids produced. At the outset we assumed that bile did accelerate autolysis, but were skeptical of enzyme activation. Our first experiment was designed therefore specifically to determine whether activation took place by following the rate of digestion of some foreign digestible protein like gelatin (see Table VI). To our surprise we found that bile does not significantly change the rate of autolysis of such tissues as liver, heart, spleen, kidney, and thymus. It cannot function therefore either as an activator or as a coenzyme in the autolytic mechanism.

Experiment I. The Effect of Bile on Autolysis of Various Tissues.—The tissues were ground, sieved, and made up to 250 cc. as in previous experiments. Bile was present where indicated in the tables. Digestion was estimated by formol titration of the amino-acids in the trichloroacetic acid filtrates. Bile caused marked alterations in the appearance of the protein precipitates, but did not interfere seriously with the end-points of titrations. The figures in the tables representing digestion are given in cc. of 0.2 N NaOH per 25 cc. of filtrates.

TABLE I.

Pig Liver; Mixed Pig and Beef Bile.

	Days.				Gain.
	0	2	5	16	
I. Control	0.40	2.35	2.90	3.45	3.05
II. " + 100 cc. bile	0.45	1.15	1.55	2.10	1.55
III. " + 25 " "	0.45	1.80	2.30	3.05	2.60
IV. " + 6 " "	0.50	2.10	2.80	3.45	2.95

In this experiment we find definite inhibition of autolysis in the higher concentrations of the bile.

[3] Bradley, H. C., and Taylor, J., *J. Biol. Chem.*, 1916, xxv, 261.

TABLE II.

Pig Heart; Pig Kidney; Beef Bile.

	Days 0	2	5	16	Gain
I. Control, heart muscle ..	0 30		0.80	1 05	0 75
II. " + 100 cc. bile	0 35	0 60	0 65	0 90	0 55
III. " + 25 " "	0 35	0 65	0 85	1 10	0 75
IV. " + 6 " "	0 30		0 85	1 20	0 85
V. " in 0.02 N HCl	0 30	0 95	1 40	1.95	1 65
I. Control, kidney	0 50	0 95	1 00	1 25	0 75
II. " + 100 cc. bile	0 60	1 00	1 35	1 80	1 20
III. " + 25 " " ...	0 50	1 00	1 30	1 80	1 30
IV. " + 6 " "	0 50	1 00	1 15	1 30	0 80
V. " in 0.02 N HCl.....	0 50		3 05	3 70	3.20

Bile has no significant effect on cardiac muscle. The stronger solutions appear to inhibit slightly, the weaker to accelerate. Acidity produces definite increase of autolysis. In the case of the kidney, higher concentrations of bile appear to accelerate the autolysis and lead to a higher final level of equilibrium. The effect is of little significance, however, when compared with the 300 per cent increase of autolysis in the presence of acid. This small increase of autolysis in the presence of bile we have found to be the rule with kidney and thymus, and it is sometimes found in the spleen. It clearly has no relation to the twentyfold rate of cytolysis reported by Tatum over the cytolysis in the presence of acid.

The cause of this regular increase of autolysis in some tissues we thought might be accounted for by the bile proteins introduced. Precipitating the proteins from 100 cc. of bile and adding the washed precipitate to a tissue digest failed to produce a measurable effect. It is possible that the alcohol-precipitated protein was sufficiently altered to fail to digest where the protein in solution was digestible. It is more probable that there are sufficient traces of fats of the lower fatty acids in some of the tissues to produce appreciable amounts of fatty acids under the accelerating influence of bile on lipolysis. These water-soluble acids would accelerate the rate and increase the extent of proteolysis.

TABLE III.

Beef Kidney; Beef Bile.

	Days. 0	2	7	Gain.
I. Control	0.40	0.75	0.90	0.50
II. " + 100 cc. bile	0.60	1.10	1.40	0.80
III. " + 25 " "	0.45	0.90	1.15	0.70
IV. " + bile protein	0.45	0.75	0.90	0.50
V. " in 0.02 N HCl	0.45	2.75	3.45	3.00

TABLE IV.

Beef Spleen; Calf Thymus; Beef Bile.

	Days. 0	2	7	16	Gain.
I. Control, beef spleen	0.45	2.15	2.55	2.80	2.35
II. " + 100 cc. bile	0.60	1.80	2.50	3.10	2.50
III. " + 25 " "	0.55	2.10	2.75	3.15	2.60
IV. " + 6 " "	0.55	2.30	2.70	3.10	2.45
V. " in 0.02 N HCl	0.60	3.10	3.95	4.10	3.50
I. Control, calf thymus	0.75	1.40	1.90	2.10	1.35
II. " + 100 cc. bile	0.70	1.55	2.25	2.70	2.00
III. " + 25 " "	0.70	1.60	2.25	2.70	2.00
IV. " + 6 " "	0.70	1.45	2.10	2.50	1.80
V. " in 0.02 N HCl	0.75	2.60	3.15	3.40	2.65

TABLE V.

Beef Spleen; Calf Thymus; Beef Bile.

	Days. 0	3	7	Gain.
I. Control, beef spleen	0.50	1.75	2.20	1.70
II. " + 100 cc. bile	0.75	2.00	2.35	1.60
III. " + 25 " "	0.65	1.95	2.30	1.65
IV. " in 0.02 N HCl	0.55	3.50	3.65	3.10
I. Control, calf thymus	0.40	1.10	1.35	0.95
II. " + 100 cc. bile	0.60	1.30	1.85	1.25
III. " + 25 " "	0.45	1.20	1.65	1.20
IV. " in 0.02 N HCl	0.50	2.65	3.40	2.90

Comparing Tables IV and V we see that in one case the spleen is slightly accelerated and increased in its autolysis by bile, in the other the reverse is true. If the phenomenon were one involving activation we should look for uniformity. Its variability suggests some factor like fat or ester content which is likely to vary under different nutritional conditions.

Experiment II. Bile and the Digestion of Foreign Proteins.—Gelatin was chosen as a typical foreign protein normally digested by the liver proteases. Activation of the enzymes should lead to a more rapid hydrolysis of gelatin.

TABLE VI.

Pig Liver; Gelatin; Dried Bile.

	Time.						Gain.
	0	4 hrs.	11 hrs.	1 day.	6 days.	14 days.	
I. Control	0.55	0.90	1.20	1.50	2.70	2.90	2.35
II. " + gelatin	0.60	1.10	1.50	2.00	3.55	3.90	3.30
III. " + 20 gm. bile	0.90	1.15	1.30	1.45	2.20	2.50	1.60
IV. Control + bile + gelatin	0.95	1.20	1.40	1.65	2.50	2.90	1.95
V. Control + 5 gm. bile	0.70	0.90	1.10	1.40	2.30	2.50	1.80
VI. Control + bile + gelatin	0.80	1.05	1.35	1.70	2.80	3.10	2.30
VII. Control in 0.02 N HCl	0.55	1.15	1.70	2.10	3.45	3.70	3.15
VIII. Control + gelatin in HCl	0.65	1.15	1.80	2.60	4.20	4.55	3.90

Analysis of the figures shows the following.

Gelatin in the control digests 0.95 cc.
" with 20 gm. of bile present digests 0.35 cc.
" " 5 " " " " " 0.50 "
" " HCl present digests 0.75 cc.

It is clear that bile retards the digestion of gelatin, as it does the digestion of the liver proteins. There is no suggestion of an activation phenomenon.

DISCUSSION.

The experiments emphasize the caution which must be exercised in interpreting histological changes in terms of chemical reactions without at the same time demonstrating the presence of such reactions. It is evident, too, from the combined data presented by Tatum and by us that a sharp distinction must be made between autolysis and cytolysis. The disintegration of cell structures may take place without accompanying autolytic changes, as when the red corpuscles are laked with water, bile salts, saponin, or toluene. It may take place with no disturbance of the autolytic mechanism, except perhaps to hasten the death of the tissue and so the onset of autolysis. Cytolysis of this kind is described in Tatum's paper—blocks of fresh liver, spleen, kidney, etc., immersed in bile. Finally cytolysis may be the direct result of autolysis, as when postmortem changes occur in the same tissues under normal conditions, or where blocks of these tissues are immersed in acid of the proper concentration to accelerate autolysis.

The mechanism of bile cytolysis is not clear in the cases presented by Tatum beyond the fact that it is not correlated with an increased rate of autolysis. It may be due to the solvent effect of the bile salts upon the cell lipoids. It may be due to the greater dispersion of structure colloids in the lowered surface tension of bile solutions, and thus have an apparent solvent effect. It is well known that bile dissolves the visual purple of the retina as a more highly dispersed colloid, and the two phenomena are not unlike.

SUMMARY.[4]

1. Autolysis does not parallel the rapid cytolysis of tissues immersed in bile or its salts. Bile does not accelerate the autolysis of liver, spleen, kidney, thymus, and heart muscle to a significant degree.

[4] We have submitted our manuscript to Dr. Tatum for his comment, and find that he has independently been engaged in much the same line of experimentation. His results, too, are in substantial agreement with those presented here.

2. Bile does not activate the enzymes associated with the phenomena of autolysis in the tissues investigated, nor does it act as a coferment.

3. The cytolytic effect of bile on these tissues must therefore be a process distinct from autolysis.

THE USE OF COTTON SEED AS FOOD.*

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(Received for publication, January 17, 1917.)

The following quotations from recent literature relating to the use of cotton seed products as food serve to illustrate the general problem of so called cotton seed meal injury or toxicity:

"Cotton seed meal is one of the most valuable feedstuffs at the command of the American stockman. After the animal has digested it, the value of the residue as fertilizer is about three-fourths the original value of the meal. The United States uses only part of the cotton seed meal which it produces, and one of the reasons which prevent a larger domestic consumption of this by-product of the cotton industry is the danger that sickness and death may follow its use. Cattle fed for more than 90 to 120 days on a heavy cotton seed meal ration (6 pounds or more per head daily) become lame, and their eyes discharge freely, blindness often resulting. Deaths may occur, especially in young animals. Pigs are peculiarly susceptible to the effects of cotton seed meal, possibly because they are usually fed a larger quantity of the meal in proportion to their body weight. In feeding pigs, symptoms of sickness may appear at any time after 3 weeks of feeding, and deaths frequently occur with little warning. Various systems of feeding cotton seed meal to pigs have been devised. Some of them appear to minimize its danger somewhat, but none of them prevent it entirely. This product, therefore, can not be regarded as a safe feed for pigs in the combinations in which it has heretofore usually been fed."[1]

Referring to the experiments in feeding cotton seed meal which

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Rommel, G. M., and Vedder, E. B., *J. Agric. Research*, 1915, v, 489.

have been made by various agricultural station workers, Wells and Ewing[2] summarize:

"The results of such experiments do not agree entirely and few absolute conclusions can be drawn from them. They indicate, however, that swine—particularly young pigs—calves, sheep, horses, cows, steers, dogs, cats, guinea pigs, rabbits, fish, poultry, and other animals may be injured by eating cotton seed meal. Some of the smaller animals, such as pigs and calves, seem to be more susceptible to its injury than cows, steers, and similar animals. This, however, may have been due to their youth, or, more probably, to a consumption of larger quantities of meal in proportion to their live weight. When the meal was fed in connection with pasturage, or when it had been steamed, boiled, or fermented, or when fed with mineral matter, particularly iron compounds, it often seemed to exert no apparent injury to pigs even when fed in rather large quantities. The injury resulting from the feeding of cotton seed meal to stock has been attributed to: (a) the oil in the meal; (b) its crude fiber; (c) excess of nitrogen and perverted metabolism; (d) the action of bacteria and molds; (e) presence of betain, cholin, or other alkaloids, and to gossypol; (f) to injurious phosphorous compounds; (g) to a protein group containing ly bound sulphur, which interferes with normal iron metabolism; to worms and certain other causes, which, perhaps, are not of sufficient importance to merit discussion here."

Until quite recently there has been little, if any, appreciation of the possibility that so called cotton seed injury may be associated with a lack of some essential dietary component rather than attributable solely or even in part to the presence of a positively harmful ingredient of the seed. According to present day criteria a "balanced" or "physiological" ration must represent something more than an adequate quota of calories including no less than a certain minimal proportion of protein, along with inorganic salts and, perhaps, "roughage." The protein must be suitable in quality, so as to furnish a sufficient yield of all the needed amino-acids; the inorganic nutrients must be both quantitatively and qualitatively appropriate; and evidently certain vitamines (including those soluble in some of the natural fats, *e.g.*, butter fat or cod liver oil, and also those soluble in water which appear to be widely distributed in the active cells of plants and animals) are needed, even if the absolute quantity requisite

[2] Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station*, *Bull.* 119, 1916.

STUDIES OF AUTOLYSIS.

V. THE INFLUENCE OF BILE ON AUTOLYSIS.

By H. C. BRADLEY and JOSEPH TAYLOR.

n the Laboratory of Physiological Chemistry, University of Wisconsin, Madison.)

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TABLE II.

Pig Heart; Pig Kidney; Beef Bile.

	Days.				Gain
	0	2	5	16	
I. Control, heart muscle	0.30		0.80	1.05	0.75
II. " + 100 cc. bile	0.35	0.60	0.65	0.90	0.55
III. " + 25 " "	0.35	0.65	0.85	1.10	0.75
IV. " + 6 " "	0.30		0.85	1.20	0.85
V. " in 0.02 N HCl	0.30	0 95	1.40	1.95	1.65
I. Control, kidney	0 50	0.95	1.00	1.25	0.75
II. " + 100 cc. bile	0.60	1.00	1.35	1.80	1.20
III. " + 25 " "	0.50	1.00	1.30	1.80	1.30
IV. " + 6 " "	0.50	1.00	1.15	1.30	0 80
V. " in 0.02 N HCl	0.50		3.05	3.70	3.20

Bile has no significant effect on cardiac muscle. The stronger solutions appear to inhibit slightly, the weaker to accelerate. Acidity produces definite increase of autolysis. In the case of the kidney, higher concentrations of bile appear to accelerate the autolysis and lead to a higher final level of equilibrium. The effect is of little significance, however, when compared with the 300 per cent increase of autolysis in the presence of acid. This small increase of autolysis in the presence of bile we have found to be the rule with kidney and thymus, and it is sometimes found in the spleen. It clearly has no relation to the twentyfold rate of cytolysis reported by Tatum over the cytolysis in the presence of acid.

The cause of this regular increase of autolysis in some tissues we thought might be accounted for by the bile proteins introduced. Precipitating the proteins from 100 cc. of bile and adding the washed precipitate to a tissue digest failed to produce a measurable effect. It is possible that the alcohol-precipitated protein was sufficiently altered to fail to digest where the protein in solution was digestible. It is more probable that there are sufficient traces of fats of the lower fatty acids in some of the tissues to produce appreciable amounts of fatty acids under the accelerating influence of bile on lipolysis. These water-soluble acids would accelerate the rate and increase the extent of proteolysis.

TABLE III.

Beef Kidney; Beef Bile.

	Days.			Gain.
	0	2	7	
I. Control	0.40	0.75	0.90	0.50
II. " + 100 cc. bile	0.60	1.10	1.40	0.80
III. " + 25 " "	0.45	0.90	1.15	0.70
IV. " + bile protein	0.45	0.75	0.90	0.50
V. " in 0.02 N HCl	0.45	2.75	3.45	3.00

TABLE IV.

Beef Spleen; Calf Thymus; Beef Bile.

	Days.				Gain.
	0	2	7	16	
I. Control, beef spleen	0.45	2.15	2.55	2.80	2.35
II. " + 100 cc. bile	0.60	1.80	2.50	3.10	2.50
III. " + 25 " "	0.55	2.10	2.75	3.15	2.60
IV. " + 6 " "	0.55	2.30	2.70	3.10	2.45
V. " in 0.02 N HCl	0.60	3.10	3.95	4.10	3.50
I. Control, calf thymus	0.75	1.40	1.90	2.10	1.35
II. " + 100 cc. bile	0.70	1.55	2.25	2.70	2.00
III. " + 25 " "	0.70	1.60	2.25	2.70	2.00
IV. " + 6 " "	0.70	1.45	2.10	2.50	1.80
V. " in 0.02 N HCl	0.75	2.60	3.15	3.40	2.65

TABLE V.

Beef Spleen; Calf Thymus; Beef Bile.

	Days.			Gain.
	0	3	7	
I. Control, beef spleen	0.50	1.75	2.20	1.70
II. " + 100 cc. bile	0.75	2.00	2.35	1.60
III. " + 25 " "	0.65	1.95	2.30	1.65
IV. " in 0.02 N HCl	0.55	3.50	3.65	3.10
I. Control, calf thymus	0.40	1.10	1.35	0.95
II. " + 100 cc. bile	0.60	1.30	1.85	1.25
III. " + 25 " "	0.45	1.20	1.65	1.20
IV. " in 0.02 N HCl	0.50	2.65	3.40	2.90

Comparing Tables IV and V we see that in one case the spleen is slightly accelerated and increased in its autolysis by bile, in the other the reverse is true. If the phenomenon were one involving activation we should look for uniformity. Its variability suggests some factor like fat or ester content which is likely to vary under different nutritional conditions.

Experiment II. Bile and the Digestion of Foreign Proteins.—Gelatin was chosen as a typical foreign protein normally digested by the liver proteases. Activation of the enzymes should lead to a more rapid hydrolysis of gelatin.

TABLE VI.

Pig Liver; Gelatin; Dried Bile.

	Time.						Gain.
	0	4 hrs.	11 hrs.	1 day.	6 days.	14 days.	
I. Control.............	0.55	0.90	1.20	1.50	2.70	2.90	2.35
II. " + gelatin...	0.60	1.10	1.50	2.00	3.55	3.90	3.30
III. " + 20 gm. bile...............	0.90	1.15	1.30	1.45	2.20	2.50	1.60
IV. Control + bile + gelatin............	0.95	1.20	1.40	1.65	2.50	2.90	1.95
V. Control + 5 gm. bile...............	0.70	0.90	1.10	1.40	2.30	2.50	1.80
VI. Control + bile + gelatin............	0.80	1.05	1.35	1.70	2.80	3.10	2.30
VII. Control in 0.02 N HCl..............	0.55	1.15	1.70	2.10	3.45	3.70	3.15
VIII. Control + gelatin in HCl............	0.65	1.15	1.80	2.60	4.20	4.55	3.90

Analysis of the figures shows the following.

Gelatin in the control digests 0.95 cc.
" with 20 gm. of bile present digests 0.35 cc.
" " 5 " " " " " 0.50 "
" " HCl present digests 0.75 cc.

It is clear that bile retards the digestion of gelatin, as it does the digestion of the liver proteins. There is no suggestion of an activation phenomenon.

DISCUSSION.

The experiments emphasize the caution which must be exercised in interpreting histological changes in terms of chemical reactions without at the same time demonstrating the presence of such reactions. It is evident, too, from the combined data presented by Tatum and by us that a sharp distinction must be made between autolysis and cytolysis. The disintegration of cell structures may take place without accompanying autolytic changes, as when the red corpuscles are laked with water, bile salts, saponin, or toluene. It may take place with no disturbance of the autolytic mechanism, except perhaps to hasten the death of the tissue and so the onset of autolysis. Cytolysis of this kind is described in Tatum's paper—blocks of fresh liver, spleen, kidney, etc., immersed in bile. Finally cytolysis may be the direct result of autolysis, as when postmortem changes occur in the same tissues under normal conditions, or where blocks of these tissues are immersed in acid of the proper concentration to accelerate autolysis.

The mechanism of bile cytolysis is not clear in the cases presented by Tatum beyond the fact that it is not correlated with an increased rate of autolysis. It may be due to the solvent effect of the bile salts upon the cell lipoids. It may be due to the greater dispersion of structure colloids in the lowered surface tension of bile solutions, and thus have an apparent solvent effect. It is well known that bile dissolves the visual purple of the retina as a more highly dispersed colloid, and the two phenomena are not unlike.

SUMMARY.[4]

1. Autolysis does not parallel the rapid cytolysis of tissues immersed in bile or its salts. Bile does not accelerate the autolysis of liver, spleen, kidney, thymus, and heart muscle to a significant degree.

[4] We have submitted our manuscript to Dr. Tatum for his comment, and find that he has independently been engaged in much the same line of experimentation. His results, too, are in substantial agreement with those presented here.

2. Bile does not activate the enzymes associated with the phenomena of autolysis in the tissues investigated, nor does it act as a coferment.

3. The cytolytic effect of bile on these tissues must therefore be a process distinct from autolysis.

THE USE OF COTTON SEED AS FOOD.*

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The following quotations from recent literature relating to the use of cotton seed products as food serve to illustrate the general problem of so called cotton seed meal injury or toxicity:

"Cotton seed meal is one of the most valuable feedstuffs at the command of the American stockman. After the animal has digested it, the value of the residue as fertilizer is about three-fourths the original value of the meal. The United States uses only part of the cotton seed meal which it produces, and one of the reasons which prevent a larger domestic consumption of this by-product of the cotton industry is the danger that sickness and death may follow its use. Cattle fed for more than 90 to 120 days on a heavy cotton seed meal ration (6 pounds or more per head daily) become lame, and their eyes discharge freely, blindness often resulting. Deaths may occur, especially in young animals. Pigs are peculiarly susceptible to the effects of cotton seed meal, possibly because they are usually fed a larger quantity of the meal in proportion to their body weight. In feeding pigs, symptoms of sickness may appear at any time after 3 weeks of feeding, and deaths frequently occur with little warning. Various systems of feeding cotton seed meal to pigs have been devised. Some of them appear to minimize its danger somewhat, but none of them prevent it entirely. This product, therefore, can not be regarded as a safe feed for pigs in the combinations in which it has heretofore usually been fed."[1]

Referring to the experiments in feeding cotton seed meal which

* The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington, D. C.

[1] Rommel, G. M., and Vedder, E. B., *J. Agric. Research*, 1915, v, 489.

have been made by various agricultural station workers, Wells and Ewing[2] summarize:

> "The results of such experiments do not agree entirely and few absolute conclusions can be drawn from them. They indicate, however, that swine—particularly young pigs—calves, sheep, horses, cows, steers, dogs, cats, guinea pigs, rabbits, fish, poultry, and other animals may be injured by eating cotton seed meal. Some of the smaller animals, such as pigs and calves, seem to be more susceptible to its injury than cows, steers, and similar animals. This, however, may have been due to their youth, or, more probably, to a consumption of larger quantities of meal in proportion to their live weight. When the meal was fed in connection with pasturage, or when it had been steamed, boiled, or fermented, or when fed with mineral matter, particularly iron compounds, it often seemed to exert no apparent injury to pigs even when fed in rather large quantities. The injury resulting from the feeding of cotton seed meal to stock has been attributed to: (*a*) the oil in the meal; (*b*) its crude fiber; (*c*) excess of nitrogen and perverted metabolism; (*d*) the action of bacteria and molds; (*e*) presence of betain, cholin, or other alkaloids, and to gossypol; (*f*) to injurious phosphorous compounds; (*g*) to a protein group containing loosely bound sulphur, which interferes with normal iron metabolism; to worms and certain other causes, which, perhaps, are not of sufficient importance to merit discussion here."

Until quite recently there has been little, if any, appreciation of the possibility that so called cotton seed injury may be associated with a lack of some essential dietary component rather than attributable solely or even in part to the presence of a positively harmful ingredient of the seed. According to present day criteria a "balanced" or "physiological" ration must represent something more than an adequate quota of calories including no less than a certain minimal proportion of protein, along with inorganic salts and, perhaps, "roughage." The protein must be suitable in quality, so as to furnish a sufficient yield of all the needed amino-acids; the inorganic nutrients must be both quantitatively and qualitatively appropriate; and evidently certain vitamines (including those soluble in some of the natural fats, *e.g.*, butter fat or cod liver oil, and also those soluble in water which appear to be widely distributed in the active cells of plants and animals) are needed, even if the absolute quantity requisite

[2] Wells, C. A., and Ewing, P. V., *Georgia Agric. Exp. Station, Bull. 119*, 1916.

is small. Furthermore, positively toxic substances must be absent or at least detoxicated in some way.

Basing their decision upon the similarity of the symptoms of the so called cotton seed poisoning of pigs with those seen in the "deficiency disease," beri-beri, Rommel and Vedder have come to the conclusion that cotton seed injury to pigs is probably due to some *deficiency* in the ration. Precisely wherein the shortcoming lies is not indicated by them, though they offer the following explanation:

"The grain with which the cotton seed meal is most frequently combined is corn. Corn is notoriously deficient as a single feed for animals, and it must be properly balanced to be fed satisfactorily. The excellent results in feeding pigs which can be obtained from rations of corn meal and skim milk or other animal products, such as tankage, blood meal, fish meal, etc., are out of all proportion to the facts indicated by the conventional chemical analyses of protein, carbohydrates, and fat. When corn meal is fed with cotton seed meal, a combination is made of two feeds both of which are deficient."

To avoid confusion of the questions at issue it must be noted that cotton seed products for feeding purposes are available in several commercial forms. Cotton seed *kernels* are obtained when the whole cotton seed is decorticated and freed from most of the hulls. Cotton seed *meal* is the term applied to the ground cotton seed cake from which most of the oil has been pressed. Cotton seed *flour* is prepared by finely grinding and sifting the meal, whereby the lint, hulls, etc., are removed more completely than from cotton seed meal.

According to information received from the Bureau of Chemistry at Washington two processes are commonly employed in preparing cotton seed *meal*. In the first process the seeds are decorticated, ground, and then subjected in kettles to the action of live steam for about $\frac{3}{4}$ of an hour. The hot mass is freed from most of the oil by means of hydraulic presses and the resulting press cake is then ground to a meal. In the second process the oil is expressed from the seeds by means of Anderson expellers whereby the meal becomes heated. The residue is then ground as in the first process. The second procedure is frequently called the cold process. We are informed that nearly all of the mills in this country use the method employing live steam; and we do

not know of any mill at present operating which does not use heat in some form. Heat is regarded as necessary in order to obtain satisfactory yields of oil.

Marchlewski[3] isolated from the "foots" from cotton seed oil a substance which he named gossypol. Withers and Carruth[4] have isolated this from cotton seed *kernels*, by extraction with fat solvents, and they report it to be highly toxic to rabbits, guinea pigs, rats, and pigs. They state that the ether-extracted *kernels* are rendered non-toxic by removal of gossypol. According to Withers and Carruth, "cotton seed meal and flour were found toxic to rabbits but the flour produced no ill effects on rats." The unlike toxicity to rats is explained by the variation in alteration or removal of gossypol in the manufacture of the *meal* from the *kernels*. Withers and Carruth believe that the deficiency theory will not explain the quick deaths of pigs and other animals fed on cotton seed *meal*, although with all other dietary factors adequate, the toxicity may be more or less overcome.

Employing the technique which we have followed for some years in our nutrition experiments with rats, Richardson and Green[5] have undertaken an investigation on the efficiency of cotton seed meal and cotton seed flour as a food for promoting growth, development, and reproduction of the albino rat. Their results led them to conclude that failures to grow or be maintained on diets containing either cotton seed meal or cotton seed flour as the chief or sole source of protein were in each instance attributable to a lack of some (non-protein) essential, such as certain inorganic salts or the growth-promoting substances present in butter fat and the water-soluble vitamine of milk, rather than to any apparent toxicity. When butter fat and "protein-free milk" or whole milk powder, which also contains the vitamines in question, were added to cotton seed flour, satisfactory nutrition was maintained over long periods. Albino rats were still alive after nearly a year on diets containing as much as 33 to 50 per cent cotton seed flour. Richardson and Green state that "animals fed on cotton seed meal extracted with ether, according to the

[3] Marchlewski, L., *J. prakt. Chem.*, 1899, lx, 84.
[4] Withers, W. A., and Carruth, F. E., *J. Agric. Research*, 1915, v, 261.
[5] Richardson, A. E., and Green, H. S., *J. Biol. Chem.*, 1916, xxv, 307.

method of Withers, have shown no nutritional advantages over those rats fed on the unextracted meal." In fact, failure sometimes occurred sooner on the extracted meal diets owing, it is suggested, to the removal of the *fat-soluble* growth-promoting vitamine such as is present in milk fat. This should be kept in mind in connection with the contradictory statement of Withers and Carruth that "cotton seed kernels are rendered less toxic by the partial extraction of gossypol and non-toxic by a more nearly complete extraction of it." Richardson and Green conclude that "cotton seed meal does not contain sufficient minerals for growth, is not actively toxic, contains efficient protein, and perhaps fat-soluble growth-promoting substances, similar to those of butter fat, but in less adequate quantities."

We are not aware that any case of injury to man has been reported from the use of cotton seed flour as food.

EXPERIMENTAL.

To ascertain whether the cotton seed proteins are, like some proteins from maize[6] notably deficient for the purposes of nutrition we have conducted feeding experiments on white rats for which cotton seed proteins furnished practically all of the food nitrogen and for which the other essential dietary components were supplied by adding to the products to be tested a suitable mixture of "protein-free milk," butter fat, and starch which, with the addition of adequate protein, has been shown in hundreds of our experiments to be sufficient for perfect growth. In this way we have found that satisfactory growth can be made by rats when either cotton seed *globulin*, or the total cotton seed protein precipitated from alkali extracts of cotton seed meal, is employed without significant amounts of other protein in the mixture.[7] The globulin was prepared by the salt extraction method[8] and represents purified protein soluble in dilute salt solution but

[6] Osborne, T. B., and Mendel, L. B., *J. Biol. Chem.*, 1913, xiv, p. xxxi; Osborne, T. B., *Science*, 1913, xxxvii, 185; Osborne and Mendel, *J. Biol. Chem.*, 1914, xvii, 325; 1914, xviii, 1; 1916, xxv, 1.

[7] The results of these and other experiments included in this paper were reported to the Society for Experimental Biology and Medicine, May 24, 1916; see *Proceedings*, 1916, xiii, 147.

[8] Osborne, T. B., and Voorhees, C. G., *J. Am. Chem. Soc.*, 1894, xvi, 778.

insoluble in water. The *total alkali-soluble protein* was prepared as follows.

Cotton seed *flour* was treated with ten times its weight of 0.4 per cent potassium hydroxide solution, filter paper pulp, which had been thoroughly extracted with the same dilute alkali, added until a thick mass resulted, and the solution pressed out with a hydraulic press. The extract was then filtered through a dense felt of paper pulp, previously washed with dilute alkali. To the clear filtrate 1 per cent hydrochloric acid was added until the protein separated sharply. The precipitate was then filtered out and washed with 50 per cent alcohol, until the washings were practically free from chlorine, digested twice with liberal quantities of 92 per cent alcohol, dehydrated with absolute alcohol, washed finally with ether, and then dried over sulfuric acid. The ash- and moisture-free preparation (dried at 110°) contained 16.98 per cent of nitrogen.

Results of feeding experiments with these products are given in the Appendix, Chart I.

In order to test the nutritive value of the total proteins as they occur in the seed, the possible lack of vitamines and inorganic salts was provided for by supplying butter fat and "protein-free milk," which we have already demonstrated to be adequate sources of all needed inorganic food ingredients and vitamines. Cotton seed *flour* was obtained from the Schulenburg Oil Mill, Schulenburg, Texas. Our samples of cotton seed *meal* were obtained by mixing samples collected for analysis by this Station and therefore were assumed to be representative of the ordinary commercial product; or they were taken from a bag of the meal purchased in the open market.

The growth curves in Chart II leave no doubt as to the adequacy of the proteins of the cotton seed *meal* or *flour* for the growth of rats. No signs of toxicity exhibited by noticeable effect on growth have been observed with any of these rations. On this diet Rat 3631 gave birth to six young which have grown vigorously.

In discussing the comparative nutritive value of different proteins in growth and the problem of the protein minimum[9] we have emphasized the fact that the comparative poverty of a protein in one or more essential amino-acid groups may not mani-

[9] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1915, xx, 351; 1915, xxii, 241; 1916, xxvi, 1.

fest itself if the supply of protein in the ration is so abundant as to be considerably in excess of the actual needs. On a more restricted plane of protein intake the "law of minimum" may manifest itself more conspicuously; accordingly, rats make quite unlike growth on the *same* (restricted) intake of such different proteins as casein and lactalbumin though they show quite similar (maximum) increments of weight when the proteins are furnished in greater abundance.

Our "standard ration" for rats contains about 16 per cent of protein in a mixture yielding about 5 calories per 1 gm. of food. With a few proteins, notably lactalbumin, almost perfect growth can be secured for a considerable period when their content in the ration is no greater than 9 per cent. Other proteins supplied in corresponding proportions exhibit their nutritive inferiority by falling behind in growth-promoting capacity. The excellent "quality" of the cotton seed proteins as a whole is attested by the satisfactory growth made on diets furnishing the equivalent of only 9 per cent of protein (N × 5.4)[10] (see Chart III, Rats 3350 and 3368). Even with 6 per cent of the protein considerable growth ensued (see Rats 3415, 3420).

The high comparative nutritive value of the cotton seed proteins was demonstrated by using cotton seed *flour* as a supplement to such decidedly inferior protein concentrates as "corn gluten,"[11] the protein of which yields only minimal quantities of lysine and tryptophane (see Chart III). Good results were likewise obtained when cotton seed *flour* was used as a supplement to distillers' grains, a commercial product with an amino-acid content not very different from that of the corn gluten; and to "vegetable albumin flour," a product rich in gliadin,—a protein deficient in lysine. Here again no failures attributable to toxicity of the cotton seed *flour* were observed (see Chart III, Rats 3514, 3523, 3515, 3525, 3569).

With regard to the presence of various vitamines in cotton seed products our experience corroborates the findings of Richard-

[10] The limitations of the method of experimentation here employed have already been discussed; see Osborne and Mendel, *J. Biol. Chem.*, 1915, xxii, 246.

[11] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1914, xviii, 1; also 1917, xxix, 69.

son and Green. When diets were prepared with "artificial protein-free milk," *i.e.*, without the water-soluble vitamines other than those contained in the cotton seed, normal gain in body weight was still possible. The food mixtures contained:

	A.	B.
	per cent	*per cent*
Cotton seed flour	41.0	
Cotton seed meal		49.1
"Artificial protein-free milk"	29.5	28.0
Butter fat	18.0	18.0
Starch	3.5	4.9
Lard	8.0	

The "artificial protein-free milk" has already been described.[12] Aside from the lactose present this consists of salts obtained from carefully purified chemicals. The mixture has been found to furnish the mineral ingredients suitable for excellent growth provided some other source of vitamines is present. Without these, prolonged growth very rarely is secured, as we have found in many trials. In the above diet the growth-promoting (fat-soluble) vitamine has been supplied by butter fat. In view of the satisfactory growths obtained with these diets in the absence of any other source of this accessory, as manifested by the records in Chart IV in the Appendix, the cotton seed must furnish the water-soluble vitamine in considerable abundance. This has now been found true of many other seeds by McCollum and his coworkers.

McCollum, Simmonds, and Pitz[13] have lately reported cotton seed *oil* prepared by ether extraction to be toxic; whereas the commercial bleached *oil* prepared by hot pressing was harmless, though without promoting growth.

The successful use of cotton seed *flour* in the ration of growing chicks was referred to in a recent paper from our laboratory.[14]

[12] Cf. Osborne and Mendel, *J. Biol. Chem.*, 1913, xv, 311; Mixture IV, described on p. 317, was used.

[13] McCollum, E. V., Simmonds, N., and Pitz, W., *Am. J. Physiol.*, 1916, xli, 361.

[14] Osborne and Mendel, *J. Biol. Chem.*, 1916, xxvi, 293.

Two of these birds (Nos. 5 and 6) beginning at the age of 28 days on a corn gluten + cotton seed flour ration gained 450 gm. (No. 5) and 556 gm. (No. 6) respectively in 79 days.

With diets containing cotton seed kernels[15] *our feeding trials have had an entirely different outcome.* All rats, both young and adult, receiving this product in the ration have promptly died within two weeks.

Here for the first time we have met with suggestions of toxicity so frequently attributed to cotton seed products. It must be noted that in the case of our cotton seed *kernel* feedings, in contrast with all other diets containing cotton seed preparations, the rats soon refused the food. This raises the question whether or not the unheated kernels contain something which causes the rats to refuse this food so that death was due to starvation rather than to any real toxicity of the cotton seed kernels. A control experiment with twelve rats (six young, six adults), deprived of all food except water, showed that these died almost as quickly as those fed with the scarcely eaten cotton seed *kernel* rations.

The statistics of the trials are given below:

*Rats on Cotton Seed Kernel Food.**

Rat.	Duration of life.	Food intake.	Original weight.	Final weight.	Loss.	
Large rats.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3246 ♀	8	40.8?	174	120	54	31
3349 ♂	7	12.3	245	183	62	25
3514 ♂	7	37.9	233	180	53	23
3516 ♂	8	19.6	300	195	105	35
3518 ♂	7	55?	174	123	51	29
3576 ♂	11	37.5?	185	114	71	38
Averages......	8	4.2 per day.	219		66	30

* The composition of the food mixture was as follows:

	per cent
Cotton seed *kernels*..............................	66
"Protein-free milk"...............................	28
Butter fat..	6

[15] These were kindly furnished by Dr. F. E. Carruth of the North Carolina Agricultural Experiment Station.

Rats on Cotton Seed Kernel Food—Concluded.

Rat.	Duration of life.	Food intake.	Original weight.	Final weight.	Loss.	
Small rats.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3672 ♀	5	4.7	86	58	28	33
3673 ♀	5	7.4	82	60	22	27
3676 ♀	6	10.0	86	64	22	26
3678 ♀	5	6.1	81	55	26	32
3679 ♀	6	7.8	80	54	26	33
3680 ♀	6	8.7	80	53	27	34
Averages.......	5.5	1.3 per day.	83		25	31

Rats without Food.

Rat.	Duration of life.	Original weight.	Final weight.	Loss.	
Large rats.					
	days	*gm.*	*gm.*	*gm.*	*per cent*
3462 ♀	6	173	115	58	34
3469 ♂	12	287	160	127	44
3472 ♂	7	247	150	97	39
3592 ♂	6	214	129	85	40
3600 ♂	6	180	108	27	40
3602 ♂	6	190	118	72	38
Averages...............	7	215		78	39
Small rats.					
3713 ♀	3	70	49	21	30
3717 ♂	4	79	50	29	37
3718 ♀	4	74	47	27	37
3720 ♀	3	69	46	23	33
3721 ♀	4	70	45	25	36
3722 ♀	4	83	54	29	35
Averages...............	4	74		26	35

A further illustration of the depressing effect of the presence of cotton seed kernels upon the food intake, with its untoward consequences upon body weight, is shown even in the experiments in which the *kernels* form a smaller fraction of the protein concentrate. When the protein, as measured by its nitrogen

content, was derived in approximately equal quantities from casein and cotton seed *kernels*, mixed together in the ration, death followed a prompt decline in the food intake, although on the previous casein ration the voluntary food intake had been sufficient for growth (see Chart V, Rats 3712, 3719).

Similar consequences resulted when cotton seed *flour* and cotton seed *kernels* were included in essentially equal proportions in the ration (see Chart V, Rat 3384). In the record of Rat 3509 (Chart V) it will be observed that the food intake promptly declined whenever cotton seed *kernels* were included in an otherwise satisfactory ration containing cotton seed *flour*. Even so little of the *kernels* as is represented by 2 per cent of protein produced an unmistakable effect, though this was by no means so marked as was the case when larger quantities of the kernels were present. Recovery, associated with increased food intake, occurred whenever the *kernels* were withdrawn from an otherwise (qualitatively) unchanged food mixture.

A number of experiment station workers who have investigated cotton seed meal as a food for the various types of farm animals have noticed the failure of these animals to eat as much of the cotton seed meal rations as they did of the other protein concentrates employed.[16]

[16] Cf. Armsby, H. P., and Hess, E. H., *Pennsylvania Agric. Exp. Station, Bull. 28*, 1894, 22;
Burkett, C. W., *North Carolina Agric. Exp. Station, Bull. 189*, 1903;
Curtis, R. S., *North Carolina Agric. Exp. Station, Bull. 215*, 1911;
Curtis, G. W., and Carson, J. W., *Texas Agric. Exp. Station, Bull. 21*, 1892;
Emery, F. E., and Kilgore, B. W., *North Carolina Agric. Exp. Station, Bull. 87*, 1892;
Georgeson, C. C., Burtis, F. C., and Otis, D. H., *Kansas Agric. Exp. Station, Bull. 53*, 1895;
Hartwell, B. L., and Lichtenthäler, R. A., *Rhode Island Agric. Exp. Station, Bull. 156*, 1914;
Hooper, J. J., and Anderson, W. C., *Kentucky Agric. Exp. Station, Bull. 176*, 1913;
Jeffrey, J. S., *North Carolina Agric. Exp. Station, Bull. 211*, 1910;
Lloyd, E. R., *Mississippi Agric. Exp. Station Report*, 1902, 17;
Nourse, D. O., *Virginia Agric. Exp. Station, Bull. 121*, 1901;
Smith, H. R., *Nebraska Agric. Exp. Station, Bull. 100*, 1907;
Vanderford, C. F., *Tennessee Agric. Exp. Station Bulls.*, 1893, vi, No. 2;
Wells, C. A., and Ewing, P. V., *J. Biol. Chem.*, 1916, xxvii, 15; *Agric. Exp. Station, Bull. 119*, 1916.

If our results are indicative of the presence of a deleterious factor in the cotton seed *kernels* in contrast with the *meal* or *flour*, the question arises as to the cause of the difference between these products. It might seem that some objectionable constituent is removed from the cotton seed when the oil is expressed from the kernels in the commercial manufacture of the meals. On the other hand, it may be that the *heating* of the kernels prior to the extraction of the oil facilitates destruction of any toxic component that is present. This appears to be the view of Withers and Carruth as expressed in personal communications to us. To obtain some answer to the questions thus raised the following series of experiments was conducted.

A sample of the ground cotton seed *kernels* which had been proved inadequate for promoting growth was pressed *without heating* in a hydraulic press for 24 hours at a pressure of about 2,000 pounds. The press cake thus obtained was ground and placed between successive layers of blotting paper and subjected to an additional pressure of 4,300 pounds for 4 hours to remove as much of the oil as possible. About 30 per cent was expressed by this method. A portion of the residue was then thoroughly extracted with ether, part of it being allowed to stand in a bottle under ether and shaken frequently for about 2 weeks, and the rest of it being extracted in a Soxhlet apparatus for about a week. The ether extract thus obtained after removal of the ether was a dark reddish brown fatty solid equal to about 10 per cent. of the press cake taken. The residual *ether-extracted cotton seed meal* was fed to two rats and proved to be entirely *adequate* as the source of protein for promoting their growth at a normal rate (see Rats 3813♂ and 3815♂, page 302). Two rats which received in their food 4.5 per cent of the *ether extract* (an amount equivalent to the quantity of residual meal which had proved to be efficient) promptly declined in weight and died in 4 and 9 days respectively (see Rats 3846 ♀ and 3853 ♀, page 302). When smaller quantities of this ether extract were fed, the rate of decline was much slower, and one rat was restored by removing the extract from the diet (see Rats 3822 ♀ and 3823 ♀, page 302). Although it is extremely difficult to extract *all* of the ether-soluble substance from the cotton seed *kernels*, it is obvious that by our

procedure something was removed from them which was either actually toxic in itself or which rendered the food so unpalatable that the rats refused to eat enough of it to sustain life.

Having demonstrated, like Withers and Carruth, that the harmful substance could be removed from the kernels by extraction with ether, the question next arose whether mere pressure would be equally efficacious. A part of the *oil* obtained by pressing the cotton seed *kernels* in the cold was heated to 110°C. for 18 hours and the rest of it left unheated. When incorporated in the food of rats neither sample was detrimental (see data on page 303). When the ration included our cold process *press cake*, still containing about 9 per cent of fat, a rapid loss of weight followed just as in feeding untreated *kernels* (see Rats 3146, 3284, 3417, 3463, 3603, page 302). In order to obtain the maximum yield of oil it is the commercial practice to heat the kernels before pressing. Samples of such *meal* which we have tested have been shown by numerous experiments to be a satisfactory food for rats.

To explain these results two hypotheses are at once suggested: either the substance which renders the *kernels* injurious is insoluble in *cold* oil and can be removed only by heating; or the injurious ingredient is actually destroyed by heat. To test the former hypothesis feeding trials were conducted in which 23 per cent of the food consisted of the crude *hot pressed oil*, obtained for us directly from the mill by Dr. Carruth. Although the food was very oily, the rats ate it readily and grew at a nearly normal rate (see Rats 3816 ♀ and 3817 ♀, page 303).

This result points to the validity of the alternative hypothesis; namely, that the injurious substance is destroyed by heat. To decide this question, samples of the untreated cotton seed *kernels* were heated for varying lengths of time under different conditions, and the resulting products incorporated in the food of rats. One sample was heated in an electric oven (dry heat) for 22 hours at 110°C. and not pressed. This treatment evidently rendered the *kernels* somewhat less injurious; for the rats fed on this sample declined more slowly than those on the *unheated kernels*, though all of them died in 11 to 24 days with an average loss of body weight amounting to 21 per cent. Another sample of 1,700 gm.

*Rats on Foods Containing Parts of the (Unheated) Cotton Seed Kernels.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
A. Cotton seed kernels, cold-pressed.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3146 ♀	13	20.4	199	138	−61	31
3284 ♀	4	9.5	185	151	−34	18
3417 ♂	13	50±	220	150	−70	32
3463 ♀	15	85±	162	100	−62	38
3603 ♀	7	8.7	138	95	−43	31
B. Cotton seed kernels, ether-extracted.						
3813 ♂	41	295.0	60	130	+70	117
3815 ♂	41	291.0	60	130	+70	117
C. Casein + ether extract of cotton seed kernels, 4.5 per cent.						
3846 ♀	4	7.1	60	41	−19	32
3853 ♀	9	23.9	61	38	−23	38
D. Casein + ether extract of cotton seed kernels, 2.25 per cent.						
3822 ♀	17	?	70	61	− 9	13
3823 ♀	18	?	65	58	− 7	11
E. Casein + ether extract of cotton seed kernels, 1 per cent.						
3813 ♂	26	168.8	130	139	+ 9	7
3815 ♂	26	161.6	130	137	+ 7	5

* The composition of the food mixtures was as follows:

	A. *per cent*	B. *per cent*	C. *per cent*	D. *per cent*	E. *per cent*
Cotton seed *kernels*.	49.5	41.5			
Ether extract of cotton seed kernels..............			4.5	2.25	1
Casein.............			18.0	18.00	18
"Protein-free milk"	28.0	28.0	28.0	28.00	28
Starch.............		2.5	29.0	29.00	29
Lard..............	4.5	10.0	2.5	4.75	6
Butter fat.........	18.0	18.0	18.0	18.00	18

*Rats on Foods Containing Cotton Seed Oil.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
Casein + cotton seed oil, cold-pressed.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3697♂	28	132.9	51	95	+44	86
3698♀	28	148.5	50	94	+44	88
3702♀	56	342.6	59	123	+64	108
3708♂	56	365.7	51	139	+88	173
3709♂	28	160.6	54	102	+48	89
Casein + cotton seed oil, cold-pressed, and heated to 110° for 18 hours.						
3699♂	16	68.0	54	60	+ 6	11
3700♂	17	82.5	49	69	+20	41
3712♂	17	103.9	50	81	+31	62
3715♀	20	107.3	55	73	+18	33
3719♀	17	110.9	60	80	+20	33
Casein + cotton seed oil, crude.						
3816♀	56	350±	66	113	+47	71
3817♀	56	338.6	60	125	+65	108

* The composition of the food mixtures was as follows:

	per cent
Casein	18
"Protein-free milk"	28
Starch	25
Butter fat	6
Cotton seed oil	23

was put in a cheese-cloth bag in the container of the hydraulic press. The apparatus was carefully insulated and steam passed into the material for $5\frac{1}{2}$ hours. The container and kernels were then quickly transferred to the hydraulic press and as much of the oil as possible was pressed out. The press cake, from which the oil was by no means completely removed, was dried at about 60° and fed to two rats.

Other samples of the ground *kernels* were subjected in jacketed containers to vigorous treatment with live steam for different lengths of time, from 1 to 6 hours. The kernels reached a tem-

perature of 104–109°C. There was some loss through distillation and spraying. The steamed kernels were dried at about 60°C. and ground.

The results of all of these experiments with the heated cotton seed kernels are tabulated below.

*Rats on Heated Cotton Seed Kernel Foods.**

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
A. Kernels, not pressed, heated for 22 hours at 110°C. in the electric oven.						
	days	*gm*	*gm.*	*gm*	*gm.*	*per cent*
3737♂	13	53.1–	67	49	–18	27
3738♂	20	58.2–	65	49	–16	25
3739♂	11	29.3	69	58	–11	16
3741♂	23	76.6	57	46	–11	19
3743♂	23	72.7	56	47	– 9	16
3744♂	24	79.3	60	45	–15	25
Averages.......	19	3.5 per day.	62		–13	21
B. Kernels, not pressed, steamed 1 hour.						
3723♀	12	27.9	54	41	–13	24
3724♂	18	75.2	50	47	– 3	6
3732♀	12	41.3	47	40	– 7	15
3734♂	12	33.8	45	40	– 5	11
3748♀	16	68.1	47	40	– 7	15
3750♀	14	47.7	50	40	–10	20
Averages.......	14	3.4 per day.	49		– 8	15
C. Kernels, partially pressed, steamed 5½ hours.						
3836♀	42	321.6	60	133	+73	122
3851♀	42	310.8	70	121	+51	73
Averages......	42	7.5 per day.	65		+62	98

Rats on Heated Cotton Seed Kernel Foods.—Concluded.

Rat.	Duration of experiment.	Food intake.	Original weight.	Final weight.	Change in weight.	
D. Kernels, not pressed, steamed 2 hours.						
	days	*gm.*	*gm.*	*gm.*	*gm.*	*per cent*
3860 ♀	28	186.8	75	108	+33	44
3863 ♂	28	163.3	57	82	+25	44
3874 ♀	28	204.7	72	119	+47	65
3875 ♂	28	206.5	61	112	+51	84
3878 ♂	28	221.6	60	126	+66	110
3893 ♀	28	178.5	58	98	+40	69
Averages.......	28	6.9 per day.	64		+44	70
E. Kernels, not pressed, steamed 4 hours.						
3861 ♀	28	209.0	75	118	+43	57
3864 ♂	28	163.4	54	89	+35	65
3876 ♂	28	203.9	66	113	+47	71
3882 ♀	28	156.7	55	92	+37	67
3888 ♂	28	192.1	55	90	+35	64
3894 ♀	28	159.4	50	81	+31	62
Averages.......	28	6.5 per day.	59		+38	64
F. Kernels, not pressed, steamed 6 hours.						
3862 ♀	28	163.2	65	81	+16	25
3866 ♂	28	152.6	57	86	+29	51
3877 ♂	28	159.3	55	83	+28	51
3887 ♀	28	158.1	63	82	+19	30
3889 ♂	28	136.3	55	69	+14	25
3892 ♀	28	155.0	56	79	+23	41
Averages......	28	5.5 per day.	59		+22	37

* The composition of these food mixtures was as follows:

	A.	B.	C.	D.	E.	F.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
Cotton seed *kernels*..	61	66	52.5	60.5	56	59.5
"Protein-free milk".	28	28	28.0	21.0	21	21.0
Lard................			1.5		5	11.5
Butter fat..........	11	6	18.0	18.5	18	18.0

These experiments show that by *steaming* the *kernels* for a sufficient length of time, they may be rendered apparently harmless for feeding. These results which correspond in many respects to the observations of Withers and Carruth, not yet published in detail, are, however, by no means uniform. In some cases *kernels* which had been heated for longer periods of time proved to be less satisfactory as food than those subjected to less prolonged steaming. It is not inconceivable that undue heating may render the meal unpalatable or otherwise unsuitable for nutrition, in addition to destroying the original deleterious substance. These facts may help to explain the conflicting evidence regarding the alleged suitability of different samples of commercial meals. The chemical reactions by which "gossypol" or any other objectionable ingredient can be altered to render it harmless have not yet been adequately investigated. Until the effects of heating cotton seed have been more fully studied, conclusive statements on this feature cannot be made.

The following tabular summary gives the statistics of average data compiled from our feeding experiments, with cotton seed products of various descriptions, relating to the source of the deleterious factor. The compositions of the food mixtures containing these products are indicated elsewhere in the text.

The facts now available may be briefly summarized as follows.

Cotton seed *kernels* are unsatisfactory for nutrition. Such samples of cotton seed *meal* and *flour* as have been tested were valuable foods for growing rats, both when used as the sole source of protein in the food, or when used in smaller quantity to supplement other less efficient protein concentrates.

The injurious substance in the *kernels* can be removed by extraction with ether and, according to Withers and Carruth, by extraction with carbon bisulfide, chloroform, benzene, or alcohol, but not with petroleum ether or gasoline. The ether-soluble material is deleterious, either because it contains some toxic ingredient or because it renders the food containing it so unpalatable that the animals refuse to eat it. This is in accord with the results obtained by Withers and Carruth, and by McCollum, Simmonds, and Pitz.[13]

Foods containing cotton seed oil prepared by pressing the kernels in the cold, or furnished as the crude unbleached com-

Experiments with Food Mixtures Containing Cotton Seed Products. Averages.

No. of rats.	Food.	Food intake per day.	Duration of experiment.	Original weight.	Change in weight.
		gm.	*days*	*gm.*	*per cent*
6	None.	0	Death in 7 days.	215	−39
6	"	0	" " 4 "	74	−35
6	Untreated kernels.	4.2	" " 8 "	219	−30
6	" "	1.3	" " 6 "	81	−31
6	Kernels heated dry at 110° 22 hours.	2.9	" " 20 "	62	−21
6	Kernels steamed 1 hour.	3.5	" " 14 "	49	−15
6	" " 2 hours.	6.9	28	64	+70
6	" " 4 "	6.5	28	59	+64
6	" " 6 "	5.5	28	59	+37
2	" " 5½ " and partly pressed.	7.5	42	65	+98
5	Kernel residues cold-pressed.	1.8	Death in 10 days.	181	−30
5	Oil cold-pressed.	5.4	28	53	+45
5	Oil cold-pressed and heated at 110° 18 hours.	6.5	17	53	+20
2	Oil, crude.	6.1	56	63	+90
2	Kernels ether-extracted.	7.2	41	60	+117
2	Ether extract of kernels, 4.5 per cent.	2.2	Death in 7 days.	61	−35
2	Ether extract of kernels, 2.25 per cent.	?	18	68	−12
2	Ether extract of kernels, 1 per cent.	6.6	26	130	+6

mercial oil prepared by heating the kernels before pressing them, are eaten without detriment by rats.

By treatment with steam under suitable conditions the *kernels* lose their deleterious effect on rats. The variations in the results of feeding different samples of cotton seed meal, which have been reported, may be due to differences in the mode of heating which the products have experienced in their preparation.

Reverting to the question already raised as to whether so called "cotton seed injury" in the feeding of domestic animals can be classed with the deficiency diseases, it is quite possible

that, as Rommel and Vedder maintain, food mixtures lacking some of the now recognized essential ingredients of an adequate diet have been employed in the past. Our experience with rats successfully grown on cotton seed rations excludes the probability that there is ordinarily any lack of the water-soluble vitamine. Whether the quota of inorganic salts furnished in agricultural practice is always sufficient we are unable to answer. It is noteworthy, however, that we have induced young rats to double their weight at a normal rate of growth on a food mixture containing nothing except cotton seed *meal*, starch, and lard. The deleterious effects of unheated cotton seed *kernels* cannot be denied. Whether the reputed detrimental effect after feeding some of the commercial cotton seed *meals* is associated with a failure to destroy a deleterious constituent—as has been indicated above—or is attributable to unsuitable methods of feeding in some cases is still debatable. The treatment of the cotton seed so as at least to render it harmless now seems to lie within the range of ready possibilities.

APPENDIX.

CHART I. Showing normal growth of rats on rations furnishing protein in the form either of the isolated *cotton seed globulin* or the *total alkali-soluble cotton seed protein.*

The food mixtures had the following composition.

	per cent	*per cent*
Cotton seed globulin	18	
Alkali-soluble cotton seed protein		18
"Protein-free milk"	28	28
Starch	28	24
Lard	8	12
Butter fat	18	18

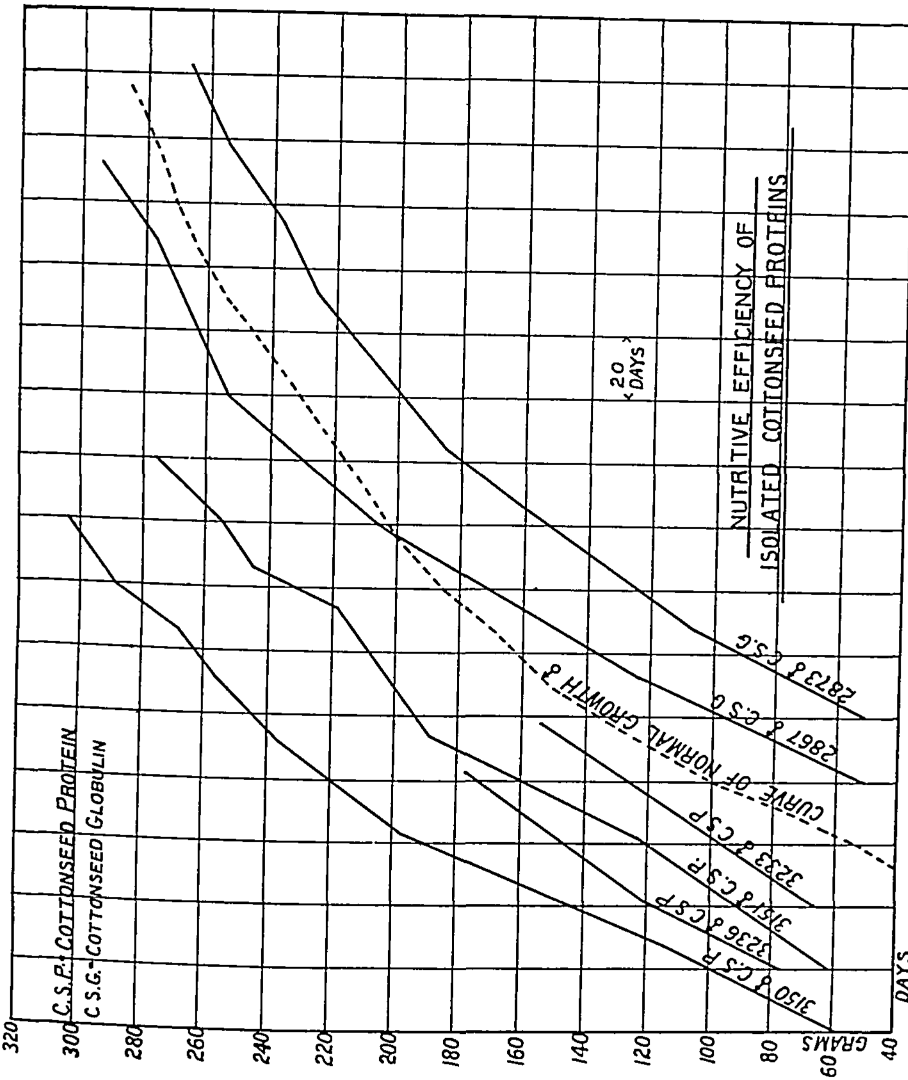
NUTRITIVE EFFICIENCY OF
ISOLATED COTTONSEED PROTEINS
C.S.P.-COTTONSEED PROTEIN
C.S.G.-COTTONSEED GLOBULIN
20 DAYS
2873 ♂ C.S.G.
2867 ♂ C.S.G.
CURVE OF NORMAL GROWTH ♂
3233 ♂ C.S.P.
3151 ♂ C.S.P.
3236 ♂ C.S.P.
3150 ♂ C.S.P.
GRAMS
DAYS
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

Chart I.

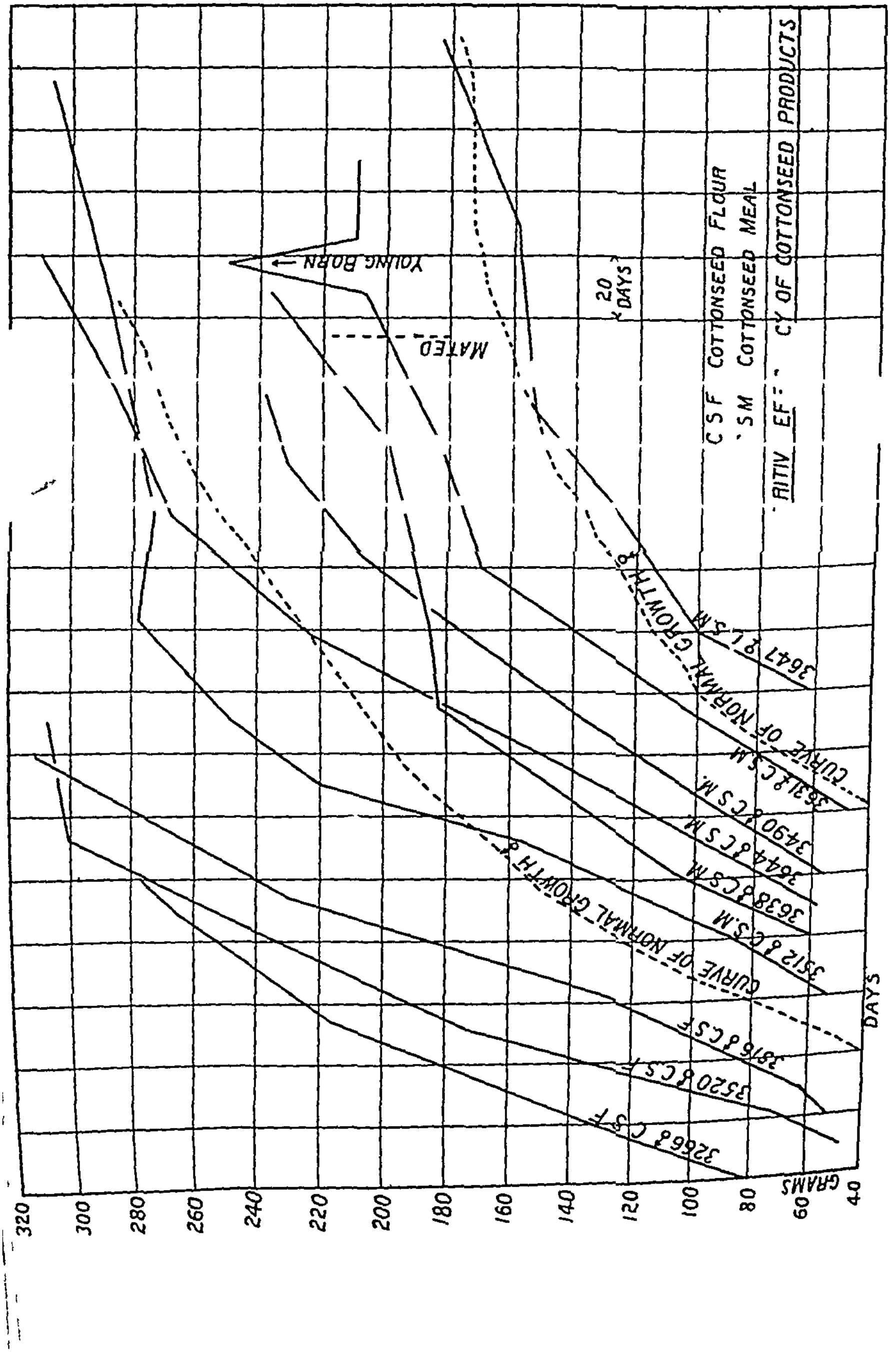
CSF COTTONSEED FLOUR
CSM COTTONSEED MEAL
YOUNG BORN
MATED
20 DAYS
CURVE OF NORMAL GROWTH
3647 C.S.M
3631 C.S.M
3490 C.S.M
3644 C.S.M
3638 C.S.M
3512 C.S.M
CURVE OF NORMAL GROWTH
3316 C.S.F
3520 C.S.F
3266 C.S.F
DAYS
GRAMS
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

CHART II. Showing the excellent growth of rats on foods in which the protein (except for the traces in the "protein-free milk") was furnished either by cotton seed *flour* or cotton seed *meal*. Suitable salts and vitamines were incorporated in the food mixtures which had the following composition.

	per cent	*per cent*
Cotton seed *flour*	41	
Cotton seed *meal*		41.0–52.5
"Protein-free milk"	28	28.0
Starch	2	0.0– 2.0
Lard	11	1.5–11.0
Butter fat	18	18.0
Approximate protein content	18	15.5–18.0

Rat 3631 ♀ mated with Rat 3644 ♂ gave birth to six young as indicated in the chart.

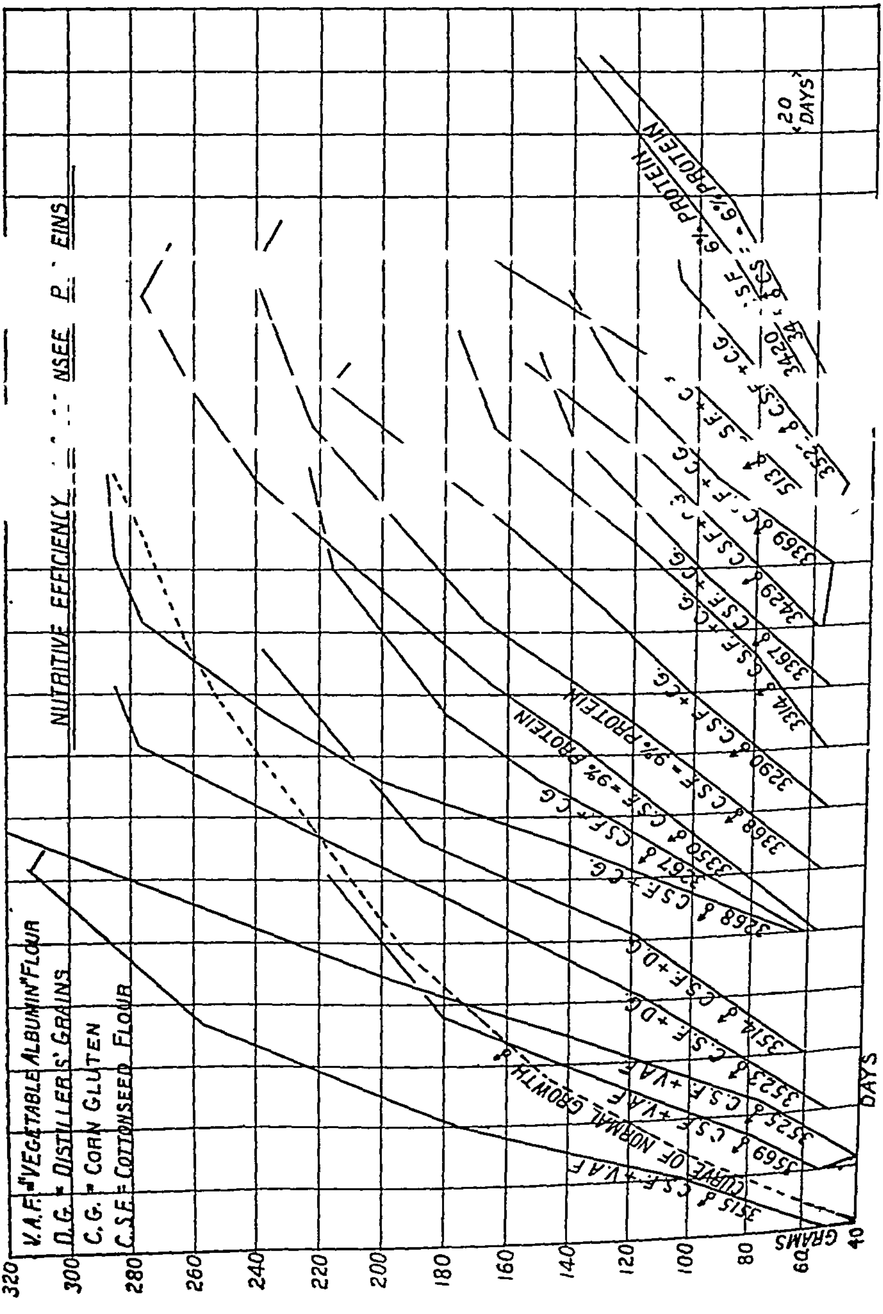
V.A.F. = "Vegetable Albumin" Flour
D.G. = Distillers' Grains
C.G. = Corn Gluten
C.S.F. = Cottonseed Flour
GRAMS
DAYS
Curve of Normal Growth

CHART III. Showing the high nutritive efficiency of cotton seed *protein* as exemplified by the excellent growth of rats upon rations containing comparatively low contents of protein in the form of cotton seed *flour* (Rats 3350, 3368, 9 per cent protein; 3415, 3420, 6 per cent protein); likewise by supplementing satisfactorily other protein concentrates—corn gluten (Rats 3267, 3268, 3290, 3314, 3367, 3369, 3429, 3513, 3522), "vegetable albumin flour" (Rats 3515, 3525, 3569), and distillers' grains (Rats 3514, 3523)—which alone failed to yield all of the amino-acids required for normal growth.

The food mixtures had the following composition.

Rats	3350, 3368.	3415, 3420.	3514, 3523.	3515, 3525, 3569.
	per cent	*per cent*	*per cent*	*per cent*
Cotton seed flour (N = 8.1 per cent)	20.5	14	14	20.5
"Vegetable albumin flour" (N = 13.1 per cent)				10.0
Distillers' grains (N = 4.7 per cent)			36	
"Protein-free milk"	28.0	28	24	28.0
Starch	27.5	30		12.5
Sucrose		4		
Lard	6.0	6	8	11.0
Butter fat	18.0	18	18	18.0

Rats	3267, 3268.	3290, 3314.	3367, 3369, 3429.	3513, 3522.
	per cent	*per cent*	*per cent*	*per cent*
Cotton seed flour (N = 8.1 per cent)	20.5	13.7	10.0	8.2
"Corn gluten" (N = 6.6 per cent)	19.0	25.3	28.5	30.4
"Protein-free milk"	28.0	28.0	28.0	28.0
Starch	3.4	6.0	6.5	7.4
Lard	11.1	9.0	9.0	8.0
Butter fat	18.0	18.0	18.0	18.0

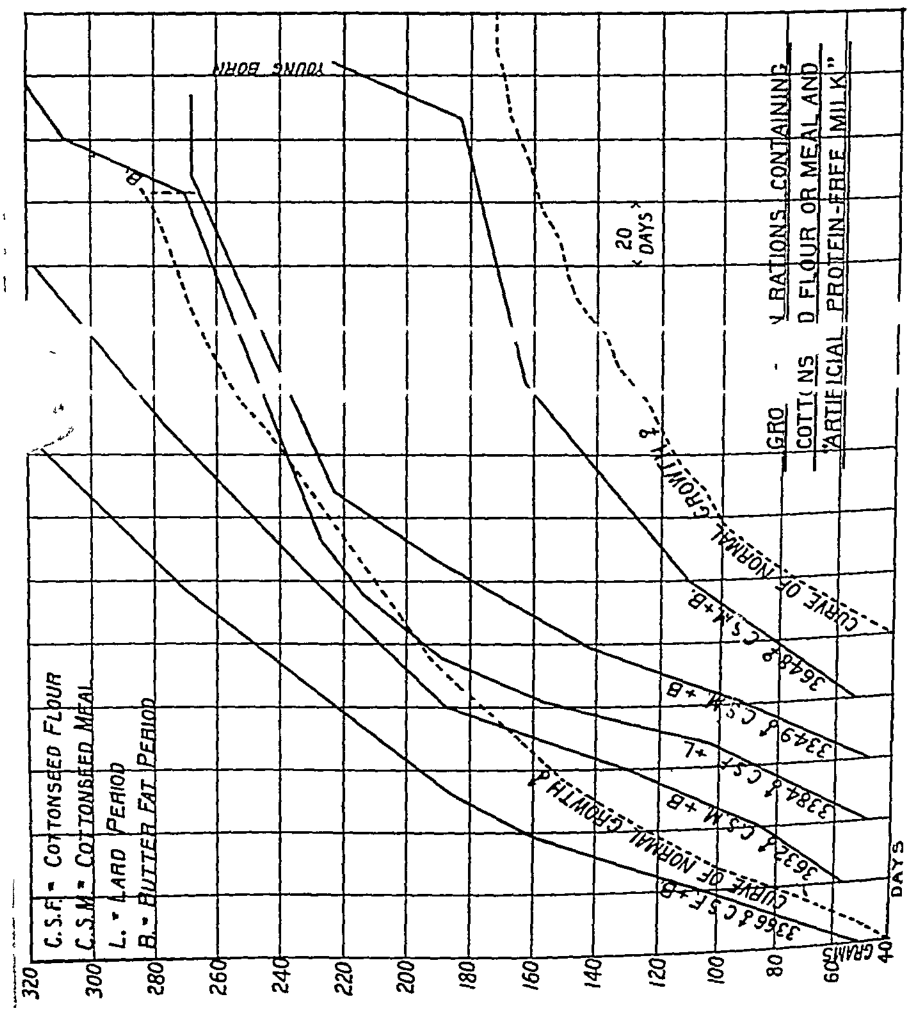
GRO RATIONS CONTAINING
COTT NS D FLOUR OR MEAL AND
"ARTIFICIAL PROTEIN-FREE MILK"
C.S.F. = COTTONSEED FLOUR
C.S.M. = COTTONSEED MEAL
L. = LARD PERIOD
B. = BUTTER FAT PERIOD
YOUNG BORN
20 DAYS
B.
CURVE OF NORMAL GROWTH ♀
CURVE OF NORMAL GROWTH ♂
3648 ♀ C.S.M.+B.
3349 ♂ C.S.M. +B
3384 ♂ C.S.F. +L
3632 ♂ C.S.M. +B
3366 ♂ C.S.F.+B.
DAYS
GRAMS
320
300
280
260
240
220
200
180
160
140
120
100
80
60
40

CHART IV. Showing growth on rations in which cotton seed *meal* or *flour* was the source of the protein and water-soluble vitamine. The satisfactory growth resulting indicates that these dietary factors are present in suitable form in these cotton seed products.

The food mixtures consisted of:

Period	Butter fat.	Lard.	Butter fat.
	per cent	*per cent*	*per cent*
Cotton seed meal	52.5		
Cotton seed flour		41.0	41.0
"Artificial protein-free milk"	28.0	29.5	29.5
Starch		1.5	3.5- 1.5
Lard	1.5	28.0	8.0-10.0
Butter fat	18.0		18.0

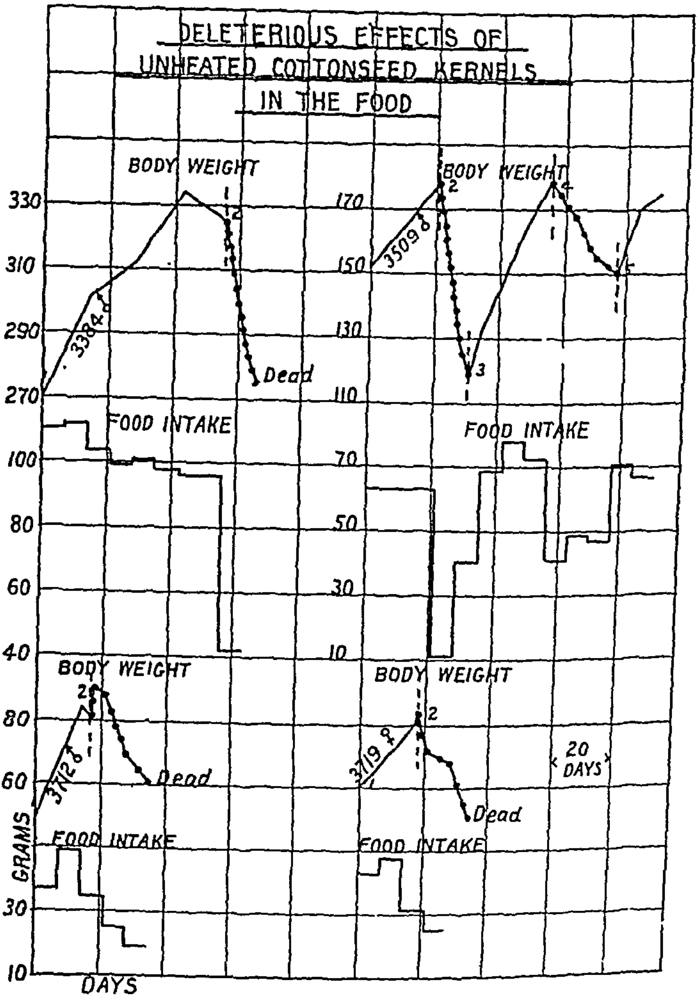
DELETERIOUS EFFECTS OF UNHEATED COTTONSEED KERNELS IN THE FOOD
BODY WEIGHT
BODY WEIGHT
330
310
290
270
170
150
130
110
3384 ♂
3509 ♂
Dead
FOOD INTAKE
FOOD INTAKE
100
80
60
40
70
50
30
10
BODY WEIGHT
BODY WEIGHT
80
60
30
10
3712 ♂
3719 ♀
Dead
Dead
20 DAYS
GRAMS
FOOD INTAKE
FOOD INTAKE
DAYS

CHART V.

CHART V. Showing the fall in body weight and decline in weekly food intake when cotton seed *kernels* were admixed in rations otherwise demonstrated satisfactory for growth. The periods during which the kernels were included in the food are indicated by the beaded line (-o—●—●—●—●-). The resumption of growth is shown in rats when the cotton seed *kernels* were excluded from the diet.

The food mixtures had the following composition.

Rats	3509.	3384.	3384.	3509.	3509
Periods	1, 3, and 5.	1.	2.	2.	4.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Cotton seed flour	26.3	41.0	13.2	13.2	21.7
Cotton seed kernels			33.0	33.0	7.7
"Protein-free milk"	28.0			28.0	28.0
"Artificial protein-free milk."		29.5	29.5		
Starch	21.7	3.5	9.3	10.8	18.6
Lard	6.0	8.0	3.0	3.0	6.0
Butter fat	18.0	18.0	12.0	12.0	18.0
Approximate protein content.	11.5	18.0	14.2	14.2	11.5

Rats	3712 and 3719.	
Periods	1.	2
	per cent	*per cent*
Casein	18	9.0
Cotton seed kernels		33.0
"Protein-free milk"	28	28.0
Starch	25	14.5
Cotton seed oil	23	
Lard		3.5
Butter fat	6	12.0

THE COMPOSITION OF ADIPOCERE.

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It is to Fourcroy (10), who in 1787 examined the waxy remains of the bodies of some children in Paris, that we owe the name adipocere. This waxy substance resulting from chemical change in buried animals has, under the names of *Leichenwachs*, *Cera cadaverica*, and Adipocere, been reported upon a number of times. Fourcroy considered it a soap of ammonia and phosphate of lime, the fats from which had a melting point of 52.5°C., and he grouped it with cholesterol and spermaceti.

Chevreul in 1812 considered a specimen of human adipocere to be a mixture of margaric and oleic acids combined with yellow odorous substances containing lime, potash, and nitrogenous matter.

Gregory (1) in 1847 made a study of hog's adipocere and concluded that it contained 75 per cent of palmitic acid, 25 per cent of stearic acid, and 2 to 3 per cent of lime. He was the first to observe the absence of glycerides, as the fat gave no acrolein reaction. The stearic acid described by him had a melting point of 79° and was obviously the mixed hydroxy stearic acids described below. He found also a small quantity of cholesterol.

Ebert (2) in 1875 found a specimen of pig's adipocere to contain about 1 per cent of ammonia and 6 per cent of insoluble residue, and the fatty acids to consist chiefly of margaric and palmitic. He found no stearic or oleic acids but recognized an acid melting at 80°C. as a hydroxy acid which he decided after analysis to be hydroxy margaric acid. This is probably the "stearic acid" of Gregory and is a mixture of two isomeric hydroxy stearic acids isolated by us from the adipocere reported in this paper.

Schmelck's (3) analyses in 1902 of three specimens, two human and one of a pig, are the only recent analyses of which we can find any record. He determined only the general physical and chemical constants of the extracted fats and fatty acids. All three gave similar analyses. Briefly his results were, melting point 62.5°C., insoluble fatty acids 83.84 per cent, ash 1.64 to 1.79, containing 84 per cent of CaO, 16 per cent of unsaponifiable matter, acid value 197, neutral value of acids 202 to 203, iodine value of acids 14.4. He made no attempt to separate the fatty acids or to study the material insoluble in ether.

Wetherill (4) some years ago, and Tarugi (5) in 1905, as the results of partial analyses, concluded that adipocere consisted chiefly of palmitic acid.

About a year ago a fine mass of adipocere was given to one of us by Mr. R. J. Blair of Ormstown, Que. It was found on a farm near Rockburn, Que., in an uncultivated wet spot, and was probably buried for 45 to 47 years. The specimen consisted chiefly of a slab 1 to 3 inches thick of the back of a pig, identified as such by some hair that remained and a portion of the jaw found with it. It weighed 15½ pounds.

The adipocere was, when cleaned from adhering earth, a hard white wax as resonant as a piece of hard paraffin, and quite homogeneous; portions taken from different parts of the mass showed almost identical physical and chemical properties. Running through the cleaned pieces fine strands of connective tissue could easily be detected.

A preliminary qualitative analysis showed that the wax was chiefly composed of ordinary saturated solid fatty acids, lime soap, fatty acids insoluble in petroleum spirit, and some connective tissue.

Quantitative Analysis.

The analyses were made on average samples taken from different parts of the mass. The finely ground material after drying *in vacuo* over sulfuric acid was first extracted in a Soxhlet with rhigolene, a very light petroleum spirit (sp. gr. 0.625, boiling point 22°C.), to separate the acids insoluble in petroleum spirit from the soluble fats and acids. The mean of a number of extractions gave 78.3 per cent soluble in rhigolene. The portion insoluble in the petroleum spirit was then extracted in a Soxhlet with ether and a further average yield of fatty acids of 15.8 per cent was obtained, leaving 5.9 per cent of insoluble residue.

The Rhigolene-Soluble Material.—This was found to be composed chiefly of palmitic with stearic and oleic acids in small quantities, together with still smaller quantities of fat and unsaponifiable matter. This mixture gave a mean melting point of 51.5–51.9°C., an acid value of 207.1, a saponification value of 210.7, or a mean molecular weight of 266.1. This last value, while it lies between those of stearic acid (284) and palmitic acid

(256), is a little high, owing to the presence of small quantities of neutral fat and unsaponifiable matter.

To obtain an idea of the quantitative relations of the constituents, we first attempted to separate the stearic acid by the Hehner and Mitchell method (6), but practically no stearic acid was obtained. It seemed from our experiments that the method is unsatisfactory and that a large excess of palmitic acid inhibits the separation of the stearic acid in alcoholic solution at 0°C. A prolonged and tedious fractional crystallization from 95 per cent alcohol was then employed to separate the acids. Six or seven series were carried through, involving a great number of separations.

The results briefly stated were: (1) The isolation of a non-saponifiable neutral substance almost insoluble in alcohol which crystallizes in rhombic spear-shaped waxy crystals giving a constant melting point of 64.5°C. This is optically inactive and gives none of the reactions of cholesterol or allied substances. The quantity obtained in a pure form (0.7 gm.) was too small to permit of accurate identification. (2) The separation of pure stearic acid to an amount corresponding to 3.3 per cent of the original adipocere. This is probably too low, as the separation of stearic acid from palmitic by fractional crystallization, or by their magnesium salts, is not accurately quantitative. (3) There was no margaric acid present. The crystals melting about 59–60°C., which occasionally formed, were eutectic mixtures of stearic and palmitic acids easily separated by varying the conditions of crystallization or by their salts into the two acids. (4) The quantity of neutral fat present, which was quite soft and pasty, was very small, only 1.37 per cent of the adipocere. This was calculated from the difference between the saponification and acid values of the mixture, and confirmed by direct extraction, after neutralization of all free fatty acids. This neutral fat gave an iodine value of 10.37, corresponding to 11.6 per cent of olein. Palmitic and oleic acids were obtained from this fat after saponification, but no stearic acid was obtained. (5) The unsaturated acids present calculated to oleic acid were 5.24 per cent of the original adipocere. These were calculated from the iodine value (Wijs' method). A liquid acid was separated from the soluble residues left from the fractional crystal-

lization, by the lead salt ether or Gusserow-Varrentrapp (7) method. Owing to the presence of some saturated acids whose lead salts were partially soluble in ether, this acid gave the low iodine value of 44.8. (6) There were only doubtful traces of volatile fatty acids.

Fatty Acids, Etc., Obtained by Ether Extraction of Adipocere.

This mixture was a solid waxy mass and contained all the fatty acids uncombined as calcium soaps together with the small quantities of fats and unsaponifiable matter. This wax gave the following results, which were determined for comparison with the constants obtained by Schmelck:

Melting point	60–63°C.
Acid value	201.7
Saponification	207
Mean mol. wt.	288
Acetyl value	34.75

The higher melting point and the lower acid and saponification values of this mixture compared with the extract by rhigolene show clearly the presence of acids of higher molecular weight.

The refractive index determined by the Abbé Zeiss refractometer was 1.4395 at 65°C.

Hydroxy Stearic Acids.

The hard waxy mixture making up the fraction insoluble in rhigolene but soluble in ether and constituting 15.8 per cent of the entire adipocere gave a melting point of 71°C. After partial purification the crystals obtained melted at 78–79°C., corresponding to the stearic acid of Gregory and to the oxy margaric acid of Ebert. They gave an acid value of 183.35, corresponding to a molecular weight of 306. (The molecular weight of hydroxy stearic acid is 300.) These crystals consisted of mixed rhombic plates and long quadratic prisms.

On careful fractional crystallization from hot ether, two well defined acids were separated, the more insoluble separating from hot ether at room temperature in prisms, often 2 cm. long, with oblique terminal planes. These when purified by recrystal-

lization melted sharply at 84.5°C. From the mother liquid by cooling to 0°C. another acid with an entirely different habit of crystallization was obtained in small glistening rhombic plates melting at 77–78°C., which on recrystallization finally melted at 78.5°C. These two acids were present in the proportion of about three of the former (melting point 84.5°C.) to two of the latter (melting point 78.5°C.).

On acetylation they proved to be monohydroxy acids. The acetyl value was obtained with difficulty and acetylation was incomplete even when boiled with acetic anhydride in presence of anhydrous sodium acetate for several hours. The acetyl value obtained was 157.2. The theoretical value (rarely attained experimentally) of monohydroxy stearic acid is 164. The filtration method described by Lewkowitsch (8) was employed. The acetyl value shows, however, that the acids were monohydroxy acids, and the molecular weight of each acid calculated from its acid value was 306, which points to monohydroxy stearic acid (mol. wt. 300) and not to hydroxy margaric (mol. wt. 284). Their silver salts gave on analysis 25.93 per cent and 25.97 per cent of silver. The theory for monohydroxy stearic acid was 26.53 per cent, and for hydroxy margaric acid 27.41 per cent.

In its general and physical properties the acid melting at 84.5° seems to be identical with a hydroxy stearic acid prepared from oleic and the so called "isooleic acid." It corresponds with the ι-hydroxy stearic acid (produced by treating a solution of oleic acid in petroleum spirit with sulfuric acid), whose constitution was established by Shukoff and Schestakoff (9) in 1903. This acid gave a refractive index of 1.439 at 90°C. Its silver salt crystallized in groups of short spear-shaped crystals. At 21°C. 100 gm. of absolute ether dissolved 2.02 gm. of the acid. 100 gm. of absolute alcohol dissolved 1.87 gm. at 0°C., and 9.17 gm. at 20.7°C. Its alcoholic solution was optically inactive.

The *other monohydroxy stearic acid crystallizing in rhombic* plates and melting at 78.5°C. is not described in the literature and may be peculiar to adipocere. This acid gave a refractive index of 1.443 at 81°C. or 1.440 when calculated to 90°C., practically identical with the ι acid (1.439). Its silver salt crystallized in long silky needles. It is much more soluble in both absolute ether and alcohol than its isomer; 100 gm. of absolute ether

dissolved 3.48 gm. of the acid at 21°C., and 100 gm. of absolute alcohol dissolved 4.41 gm. at 0°C., and 17.70 gm. at 20.7°C. Its alcoholic solution showed no optical activity.

The position of the hydroxyl group in the molecule of this acid has not yet been determined. If we assume that these acids are derived from oleic acid, it seems highly probable that it is the hitherto undescribed θ-hydroxy stearic acid. It has been satisfactorily established that the double linking in the molecule of oleic acid occurs between the θ and ι carbon atoms; *i.e.*, in the middle of the molecule. Unless we allow the possibility of another oleic acid in pig's fat with double linking in a different place, it seems unlikely that the hydroxyl group could be introduced elsewhere in the acid molecule than on the θ or ι carbon atoms.

The constitution of the acid melting at 84.5°C. has been proved by Shukoff and Schestakoff to be the ι-hydroxy stearic acid; it seems probable, therefore, that its isomer has the hydroxyl attached to the other carbon atom with double linking; *viz.*, the θ atom.

The chemical relation of these acids to oleic acid is evident from the following formulas.

$$\text{Oleic acid } CH_3(CH_2)_7CH = CH(CH_2)_7COOH.$$

$$\iota\text{-Hydroxy stearic acid } CH_3(CH_2)_7\overset{\substack{OH\\|}}{C}H\ CH_2(CH_2)_7COOH.$$

$$\theta\text{-Hydroxy stearic acid } CH_3(CH_2)_7CH_2\underset{H}{\overset{\substack{OH\\|}}{C}}(CH_2)_7COOH.$$

These two waxy acids then represent a certain portion of the fluid oleic acid, resulting from hydrolysis of olein, which has become fixed through the assumption of a molecule of water by each molecule of the unsaturated oleic acid. As stated above, the mixture of these acids was noticed in adipocere by Gregory in 1847 and called stearic acid, and was also described by Ebert in 1875 under the name of oxy margaric acid. They are probably characteristic of all adipocere.

That neither of these acids was present in its lactone form was satisfactorily established. The saponification and acid values of each acid were identical.

The Residue Insoluble in Ether.

The analysis of the residue insoluble in ether gave the following percentage composition.

	per cent
Calcium, palmitate, and stearate	61.04
Calcium hydroxy stearate	13.76
Total calcium soaps	74.8
Excess CaO	0.94
Protein	11.27
Fe_2O_3	0.48
MgO	0.24
Silica, etc	1.14
Humus and undetermined	11.13

The total ash was 0.578 per cent calculated to the original adipocere. The calcium soaps were decomposed by hydrochloric acid and the dry residue containing the acids was extracted with rhigolene as before, followed by a second extraction using ether as a solvent. By this method after recrystallization it was found that 12.94 per cent of the total residue consisted of hydroxy acids and 57.2 per cent of mixed stearic and palmitic acids. The acids soluble in rhigolene gave a melting point of 65–66°C.; and an acid value of 197.2, corresponding to a mean molecular weight of 284, showed them to consist largely of stearic acid. The hydroxy acid when recrystallized gave a constant melting point of 77–78°C. and a molecular weight of 303.3 calculated from its acid value. This is the θ-hydroxy stearic acid; no prismatic crystals of the more insoluble ι isomer could be separated.

The Nitrogenous Constituents of Adipocere.

The ammonia was determined by distilling about 10 gm. in ammonia-free water with excess of alkali. The mean of three determinations which were fairly concordant gave 0.0351 per cent calculated to the original adipocere or 0.0289 per cent of N as ammonia. The total nitrogen in the adipocere was determined by the Kjeldahl method and the mean of three analyses gave 0.1778 per cent. The nitrogen from the insoluble residue was 0.1074 per cent and was calculated to protein.

These results may be tabulated thus:

	per cent
N as ammonia	0.0289
N as protein	0.1074
N soluble (by difference)	0.0415
Total N	0.1778

The above result of the study of the nitrogenous constituents of adipocere is opposed to the view that adipocere contains an appreciable quantity of ammonium soaps. The total nitrogen is chiefly derived from the fine threads of dry connective tissue embedded in the waxy mass.

SUMMARY AND CONCLUSIONS.

In this communication an attempt has been made to obtain a quantitative analysis of mature adipocere. While the results are not of the same order of accuracy that one expects in an inorganic analysis, they are as nearly exact as our known methods of separation permit (Table I).

TABLE I.

Percentage Composition of Hard Clean Adipocere Wax.

Palmitic acid	67.52
Stearic acid	3.3
Oleic acid	5.24
ι-Hydroxy stearic acid	9.48
θ-Hydroxy stearic acid	6.32
Stearin and palmitin	1.21
Olein	0.16
Unsaponified matter	0.87
Calcium soaps	4.41
Protein	0.665
Ash	0.578
Humus and undetermined	0.247

The fatty acids, traces of fats, etc., soluble in ether, constituted 94.1 per cent of the adipocere and gave the following physical and chemical constants.

TABLE II.

Physical and Chemical Constituents of the Ether-Soluble Matter.

Sp. gr. at 100°C.	0.8436
Refractive index at 65°C.	1.436
Melting point	60–63°C.
Acid value	201.7
Saponification value	207.0
Iodine value	6.04
Acetyl value	34.75

The total nitrogen present was only 0.1778 per cent, of which only 0.0289 per cent was ammonia nitrogen.

As a result of the studies recorded in this paper we are justified in concluding that adipocere is the residue of the preexisting fats of animals. It is composed almost entirely of the insoluble saturated fatty acids left after the slow hydrolysis of the fats in wet ground. The protein matter has entirely disappeared and the glycerol, soaps, etc., resulting from the hydrolysis have been carried away in aqueous solution.

The insoluble hydroxy stearic acids which are so characteristic of adipocere are probably derived from a portion of the oleic acid in the original fat by hydration.

No margaric acid or oxy margaric acid is present. Ammonia and other soluble soaps are absent; calcium soaps alone are present in small quantity in a matured specimen of adipocere.

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APPLICATIONS OF A NEW REAGENT FOR THE SEPARATION OF AMMONIA.

I. THE COLORIMETRIC DETERMINATION OF AMMONIA IN URINE.

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The direct Nesslerization methods published some time ago from this laboratory[1] were believed to meet all ordinary requirements as to speed and accuracy in the determination of ammonia, urea, etc., and it was hoped that they might remain for some time the final form for the determination of nitrogen by Nesslerization. Last fall it was found, however, that the method for the determination of ammonia cannot be made generally available for the reason that one of the necessary reagents, Merck's purified blood charcoal, is not made in this country and is not now to be obtained at any price. No other charcoal can be used, because none other abstracts creatinine. After having failed in our endeavors to find or to prepare a suitable animal charcoal having the properties of Merck's, we have been compelled to turn to other fields in our endeavor to find a suitable substitute. In the course of this search we have found a product which, though different in its action, meets our need even better than Merck's charcoal. We have accordingly modified the microchemical Nesslerization method for ammonia on the basis of this reagent.

The new product which we now use is a synthetic mineral, an "aluminate silicate," a zeolite, discovered by Gans.[2] It possesses in a superlative degree the peculiar absorptive properties characteristic of some natural zeolites, and is manufactured and used

[1] Folin, O., and Denis, W., *J. Biol. Chem.*, 1916, xxvi, 473.

[2] Gans, R., *Jahrb. k. preuss. Geol. Landesanstalt*, 1905, xxvi, 179; 1906, xxvii, 63.

on a large scale for the "softening" of water and for other industrial purposes. The crude product is sold under the trade name "Permutit."

In the early part of our investigation we used a sample of permutit which had been in our possession for several years. This product had been imported from Germany. In order to be sure that we were not again working with a product which could not be made generally available we communicated with the makers of the American permutit and obtained samples of their product.

The two products were not quite identical in their properties, and by the means at our disposal we were unable to make them identical. The makers of the product solved this part of our problem for us, and by a modified process have made a reagent which is satisfactory for our purposes.

The essential mechanical feature of this new reagent for absorbing ammonia is that it is a clean, moderately fine, insoluble powder which gives off no dust or turbid material to water, and settles, like sea sand, from water in the course of a few seconds. By virtue of this novel feature the (absorbed) ammonia can be separated by decantation from the solution (or urine) which contained it.

The removal of ammonia by this mineral reagent is not an adsorptive phenomenon. The reagent is a complex insoluble sodium salt containing active, *i.e.*, easily replaceable, sodium, and the absorption of ammonia involves the replacement of a part of this sodium by ammonia. The chemical affinity of the active group in the reagent for ammonia is remarkably strong so that under suitable conditions the exchange becomes quantitative as far as the ammonia is concerned. The reaction is a reversible one, however, and in the presence of more than small amounts of soluble sodium salts, or other electrolytes, the equilibrium reached does not represent a quantitative absorption of the ammonia. 1 gm. of the powder takes up about 13 mg. of ammonia nitrogen in the course of a few minutes' gentle agitation and in the presence of slight excess of ammonium salt (15 mg. N). Yet 2 gm. of the reagent will not quantitatively remove 1 mg. of ammonia nitrogen in the presence of 100 mg. of sodium chloride. In the presence of 20 to 25 mg. of sodium chloride the removal

of 1 mg. of ammonia nitrogen is complete and 0.5 mg. will be removed quantitatively from as much as 50 mg. of salt. Notwithstanding the balanced character of the reaction, there is therefore an adequate margin of safety when working with urine, provided that one does not attempt to work with too much ammonia.

While the chemical reaction involved in the absorption of ammonia by this reagent is apparently a reaction between a solid and a solution, it remains to be said that the solid powder contains about 20 per cent of water, and if this water of hydration is removed by heat the activity of the reagent is lost. Even gentle dry heat (100°C.) greatly reduces its activity, so that a freshly purified and rapidly dried product is less active than the same product allowed to dry at ordinary temperatures, or than the same product dried rapidly at 100°C., and allowed to "weather" for a day or two.

Ammonia is absorbed best from neutral solutions, but is also absorbed well from weakly acid solutions. The presence of much acid is not good because the reagent is dissolved by acids. In alkaline solutions ammonia is not absorbed. Indeed, the serviceability of the reagent depends fundamentally on the fact that in the presence of sodium hydroxide the absorbed ammonia is again set free. The speed with which the absorbed ammonia is liberated depends very much on the quality of the reagent and also, of course, on the fineness of the powder. Some of the samples investigated held the ammonia so firmly that we did not secure its quantitative liberation except by using hot water (60–65°). As we found that ammonia can be Nesslerized at such temperatures we thought at one time that we had better incorporate the use of warm water in our description of the method; but we have not wished to do so, partly because the use of warm water is less convenient, and partly because in less experienced hands the use of warm water would almost certainly lead to occasional breakage of the colorimeter.[3]

[3] The product is made by The Permutit Company, 30 East 42nd Street, New York. We understand that the Company is prepared to furnish the powder in purified form and in any desired degree of fineness. We recommend the powder which passes through a 60 mesh sieve, but does not pass through an 80 mesh sieve. For other purposes a somewhat coarser powder may prove more useful.

One remarkable feature of those reagents which show a strong tendency to retain a part of the absorbed ammonia is that the longer the ammonia has remained in the absorbed condition the more difficult it is to get it all out. While we believe that no reagent will be sold for use according to our method, which possesses an unusual tendency to retain the ammonia after the addition of alkali, we suggest that every one purchasing a supply of the reagent should determine for himself how long the Nesslerized mixture must stand to develop the maximum amount of color. With our reagent not less than 95 per cent of the theoretical value is obtained in 2 to 3 minutes, and substantially theoretical figures are obtained in 10 to 15 minutes, or less. If the absorbed ammonia has been left in the powder over night the liberation of the ammonia takes a little longer and a deficit of 2 to 3 per cent may remain.

An important characteristic of this reagent for the absorption of ammonia is that it does not appreciably deteriorate by being used. After washing away the Nesslerized ammonia and surplus alkali first with water, then with one portion of 2 per cent acetic acid, then once more with water, the powder remaining is just as efficient as before for the absorption of more ammonia. We have made as many as eight successive quantitative determinations of 1 mg. of ammonia with the same 2 gm. of powder, and at the end the powder was apparently in just as good condition as at the beginning.

The above mentioned repetitions were made from the standpoint of curiosity rather than for the purpose of saving the reagent. In practice it is not worth while to restore single 2 gm. charges of the powder. We either throw the powder away (though naturally not in the sink) or, after a brief rinsing, pour it into a cylinder. When so collected the powder should be digested with warm (60°C.) 1 per cent sodium hydroxide for about an hour to remove ammonia absorbed from the tap water. After rinsing away the ammonia and most of the alkali, it is treated with 2 per cent acetic acid till no longer alkaline to phenolphthalein, and after further washing with water and drying, the powder is again ready for use.

The process for the colorimetric determination of ammonia in urine by the help of the synthetic zeolite powder is as follows:

Transfer about 2 gm. of the powder to a 200 cc. volumetric flask. Add about 5 cc. of water (no more), and with an Ostwald

pipette introduce 1 or 2 cc. of urine, or with a 5 cc. pipette introduce 5 cc. of previously diluted urine (corresponding to 1 or 2 cc. of the original urine). With urines extraordinarily poor in ammonia it may be necessary to use more urine (5 cc.), but, in so far as it is practicable, it is better not to use more than 2 cc. and to employ a weaker standard (0.5 mg. of ammonia nitrogen) for the color comparison. Our reason for not wishing to use more than 2 cc. of urine is based partly on practical experience and partly on the recognition of the fact that the salts in the urine tend to prevent the ammonia absorption from being quantitative. Rinse down the added urine by means of a little water (1 to 5 cc.), and shake gently but continuously for 5 minutes. Rinse the powder to the bottom of the flask by the addition of water (25 to 40 cc.) and decant. Add water once more and decant. (In the case of urines rich in bile it is advisable to wash once or twice more.) Add a little water to the powder, introduce 5 cc. of 10 per cent sodium hydroxide, mix, then add more water until the flask is about three-fourths full. Shake for a few seconds and then add 10 cc. of Nessler's reagent, prepared according to the directions of Folin and Denis.[4] Mix, and let stand for 10 minutes or as much longer as may be convenient. Fill up to the mark with water, mix, and compare in the colorimeter with the standard. The standard should be 1 mg. or, less frequently, 0.5 mg. of ammonia nitrogen, Nesslerized in a similar manner; that is, with 5 cc. of 10 per cent sodium hydroxide and 10 cc. of the Nessler solution, and the whole diluted to a volume of 200 cc.

It will be noted that in the above Nesslerization process we use only 10 cc. of Nessler's reagent, whereas Folin and Denis in the direct Nesslerization method employ 15 cc. We have introduced this slight change largely for reasons of economy. It is not necessary to add as much as 5 cc. of 10 per cent sodium hydroxide, to set free the absorbed ammonia—1 cc. would do equally well. But 10 per cent sodium hydroxide is less costly than the Nessler reagent, and by using 5 cc. of the former 10 cc. of the latter become adequate.

If the addition of alkali and Nessler's reagent has been done carelessly so that the reagent strikes the bottom of the flask while the powder still contains either too much ammonia not yet liberated, or too much of the

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[4] Folin and Denis, *J. Biol. Chem.*, 1916, xxvi, 479, 480.

added alkali, the powder will turn reddish from the precipitated mercury-ammonia compound. This color can be almost instantly removed by the addition of a little 2 per cent potassium iodide solution, when washing the powder for the next determination.

It will be observed that in the dilution of the Nesslerized ammonia we take no account of the presence of about 2 gm. of the insoluble powder remaining in the flask. Theoretically this may introduce an error of something less than 1 per cent. Practically this is not so, however, because the concentration of ammonia remaining in the powder is greater than the concentration of ammonia in the Nesslerized supernatant solution. The process is also simplified by thus leaving the powder in the flask.

In the table are given some analytical results demonstrating the accuracy of this new procedure. The colorimetric determinations were made exactly as described above. The check determinations were made by Folin's macro aeration process, and

Ammonia Nitrogen in Urine per Liter.

No.	Urine.	New colorimetric method.			Aeration method. NH_3 N.
		Urine used.	Colorimetric reading, standard at 20 mm.	NH_3 N.	
		cc.		*gm.*	*gm*
1	Diabetic.	2	20.1	0.50	0.50
2	Much bile.	1	21.3	0.94	0.95
3	Strong $FeCl_3$ reaction.	2	17.0	0.30	0.30
4	Albumin.	2	14.5	0.69	0.71
5	Diabetic.	2	19.8	0.25	0.25
6	Strong $FeCl_3$ reaction.	2	21.4	0.47	0.47
7	" " "	2	19.2	0.26	0.26
8	Bile, trace.	1	17.4	1.15	1.19
9	Albumin.	2	27.4	0.18	0.19
10	Diabetic.	2	19.2	0.52	0.52
11	"	2	22.0	0.46	0.45
12	"	2	15.4	0.32	0.33
13	Albumin.	2	17.9	0.56	0.54
14	Bile, albumin.	1	17.8	1.12	1.10
15	Diabetic.	2	23.5	0.43	0.42
16	Albumin.	2	25.0	0.40	0.39
17	Cat urine.	0.25	25.0	1.60	1.54

titration. These latter determinations were made in duplicate and were accepted as the standard only after duplicate titrations agreed to within 0.1 cc. of 0.1 N alkali. The colorimetric determinations were not made in duplicate, and it might be added that not a single repetition was required. They were made and recorded by one of us while the other was making the aeration determinations, and of course were finished first. All the urines except the last one were pathological specimens obtained fresh from the Massachusetts General Hospital.

From time to time attempts have been made in this laboratory to make use of ordinary tap water instead of distilled water in connection with the Nesslerization methods for the determination of nitrogen. These attempts have invariably failed, because when ordinary city water is used the Nesslerized solutions become turbid. The cause of the turbidity is the presence of magnesium salts in the water. Calcium salts do not interfere unless present in amounts sufficient to yield insoluble calcium carbonate. These disturbing impurities, as well as any traces of ammonia which may be present, are readily removed by agitating the water, either by shaking or by means of an air current, in the presence of a few gm. of purified permutit powder which we use for the quantitative absorption of ammonia. The impurities are likewise removed simply by filtering the water through a layer of the powder.

THE CALCIUM CONTENT OF CEREBROSPINAL FLUID, PARTICULARLY IN TABES DORSALIS.

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In spite of the great number of researches which have been carried out to determine the composition of cerebrospinal fluid in health and disease, hardly anything has been known with regard to the content of calcium in this fluid either in normal or pathological conditions. Of the five single determinations that we have run across in the literature three represent analyses of fetal fluid, two of these being from hydrocephalus, a fourth is from a hydrocephalic adult, and a fifth represents mixed adult spinal fluid. Thus Schmidt (1) found 15.3 mg. of calcium per 100 cc. in a hydrocephalic adult, and 7.0 mg. in a hydrocephalic fetus. Yvon (2) found in the fetus 8.0 mg. of calcium, while Mestrezat (3) in a case of hydrocephalus found at autopsy 6.2 mg. The latter author also analyzed a mixture of twenty normal spinal fluids and found 7.1 mg. of calcium. All of these results represent more or less incidental determinations of this element and do not give us accurate information as to calcium content of the spinal fluid of adults during life. After death the composition of cerebrospinal fluid has been shown to alter so considerably as not to be comparable with the composition during life (4).

In view of the known importance of calcium for certain nervous mechanisms, the authors thought it worth while to determine whether any significant alteration of the calcium content of the spinal fluid could be noted in some of the more common brain and cord affections. It was also thought that the calcium content of spinal fluid as compared with that of the blood plasma would be of interest in connection with the origin and function of the spinal fluid.

In former analyses of spinal fluid large amounts of the liquid have been employed and the determination was therefore laborious. Using the method elaborated by the present authors (5) for the determination of calcium in blood and other body fluids accurate determinations can readily be carried out on from 5 to 10 cc. of spinal fluid, amounts which may be readily obtained during life. On account of its low protein content, spinal fluid is readily ashed for the determination and this procedure was used in most cases, but the deproteinization procedure is even here much more convenient and gives consistent results, within 2 per cent of ashing. Only one-fifth of the amount of picric acid used for the blood determination is needed in this case. 10 cc. of spinal fluid in a 25 cc. volumetric flask are treated with 10 cc. of water and 1 cc. of 4 per cent sodium picrate solution, and then 1 cc. of concentrated hydrochloric acid is added slowly with rotation. The flask is then made up to mark, heated in a boiling water bath for 15 minutes, cooled, filtered through high grade ashless paper, and an aliquot (usually 20 cc.) used for the determination by the permanganate titration of precipitated oxalate.

The average calcium content of the cerebrospinal fluids studied was 5.1 mg. per 100 cc. Most of the figures lie between 5.0 and 5.3 mg. The average for the cases of tabes dorsalis was 5.0. A value of 5.0 was obtained in the case of a normal man and similar values were obtained in other cases where the spinal fluid appeared normal. Only one specimen showed a variation of more than 0.3 mg. from this value. This was a marked case of paresis with a strongly positive Wassermann reaction, and very high globulin and cell content. The case showed 6.0 mg. of calcium per 100 cc., this value being confirmed by repeating the examination. We have found in several cases of syphilis that the calcium content of the blood plasma or serum is normal as to calcium content. Our data indicate also that repeated drainage of fluid from the spinal canal has little effect upon its content of calcium, although this has been claimed to increase the cryoscopic value (6).

In only one case were we able to obtain a calcium determination on spinal fluid of a patient with low blood calcium. This was in a case of uremic coma which showed a plasma value of 8.2 mg. of calcium per 100 cc., and in the spinal fluid 5.3 mg. There

was no parallel change in the composition of the spinal fluid in this respect such as has been observed for urea (7).

Calcium Content of Cerebrospinal Fluids.

No.	Sex.	Notes.	Calcium per 100 cc.	Wassermann. Blood.	Wassermann. Spinal fluid.	Cells.	Globulin.
			mg.				
1	♂	Normal man.	5.0	−	−	N*	N
2	♂	Vasomotor ataxia.	5.0	−	−	N	N
3	♀	Uremia.	5.3	−	−	N	N
4	♂	Tabes dorsalis.	5.2		+	84	+
5		1 week later.	5.1		+		
6		3 weeks “	5.2		+		
7		4 “ “	5.1		+		
8	♂	Tabes dorsalis.	4.8		+	N	N
9	♂	“ “	5.2		+4	N	N
10		4 weeks later.	5.3		+2	33	+
11	♂	Tabes dorsalis.	5.1		+	44	+
12		4 weeks later.	5.0		−	4	N
13	♂	Tabes early.	5.2		+	24	+
14	♂	“ dorsalis.	4.9		+	N	N
15	♂	“ and paresis.	4.8		+	N	N
16	♀	“ dorsalis.	5.3	+3	+2	N	N
17	♂	“	5.3		−	33	+
18	♂	Paresis.	5.1		+		
19	♂	“	5.3		+	44	+
20	♂	“	5.2	+	+4	38	+
21	♂	“	6.0	+	+4	187	++
22	♀	Ophthalmoplegia.	5.0	−	−	44	+
23	♂	Luetic hemiplegia.	5.0		2	N	N
24	♂	Amylotrophic, lateral sclerosis.	5.3	+4		N	N
25	♂	Brain abscess.	5.0	−	−		
26		Mixed sample, ashing method.	5.04				
27			5.01				
28		Mixed Sample 26, deproteinization method.	5.00				
29			5.03				

*Normal.

The calcium content of the cerebrospinal fluid thus tends to remain constant under the conditions studied. We have found this to be true for the blood under a considerable variety of patho-

logical conditions. This same constancy has been shown for the blood of cows during pregnancy and lactation (8). The calcium content of cerebrospinal fluid is approximately one-half that of the blood plasma or serum, normal values for which lie between 9 and 11 mg. per 100 cc. This marked difference in composition is probably in part due to the very low protein content of cerebrospinal fluid and also to the more alkaline reaction of this fluid. It has been found (9) that the cerebrospinal fluid showed a P_H of 8.11 as compared with an average of 7.66 for the blood of the same series of individuals. These authors also found no alteration in the reaction of the blood or cerebrospinal fluid in cases similar to those here reported by us.

CONCLUSIONS.

The calcium content of the cerebrospinal fluid of adults is very constant at about 5.0 mg. of the element in 100 cc. of fluid. This is approximately one-half the calcium content of the blood plasma or serum of similar individuals. The calcium content was determined in a number of cases of syphilis of the nervous system. Variation greater than 0.3 mg. from the value given was obtained in but a single very severe case. In tabes dorsalis an average value of 5.1 mg. was obtained. Repeated drainage did not appear to affect the composition of the fluid in respect to calcium.

The authors are indebted to Dr. Philip B. Hawk for the opportunity of carrying out this work, and to Drs. William H. Spencer and George P. Meyer for assistance.

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THE NATURE OF THE DIETARY DEFICIENCIES OF THE OAT KERNEL.*

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In order to attain optimum results in human nutrition and animal production, we must have a thorough knowledge of the supplementary dietary relationships among our naturally occurring foodstuffs. Such knowledge can be gained only through the interpretation of the results of properly planned feeding experiments, since laboratory methods for the examination of foods are not sufficiently refined to do more than point out where lie the profitable lines of study by biological methods. We have acquired in our experiments with purified foodstuffs, with and without the addition of water or alcoholic extracts of various natural foodstuffs, and with fats from various sources, what we believe to be an adequate working hypothesis as to the factors which operate in making a successful diet.[1] We have proceeded to examine individually the dietary properties of the more important natural foods as revealed by feeding them with single or multiple additions of isolated dietary components. These studies have been described elsewhere for rice,[2] wheat,[3] wheat germ,[4] and maize kernel.[5]

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] McCollum, E. V., and Davis, M., *J. Biol. Chem.*, 1915, xxiii, 181, 231. McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916, xxv, 105; *Am. J. Physiol.*, 1916, xli, 333.

[2] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 181.

[3] McCollum and Davis, *J. Biol. Chem.*, 1915, xxi, 615. Hart, E. B., and McCollum, E. V., *ibid.*, 1914, xix, 373. Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid.*, 1916, xxv, 239. McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

[5] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 153.

The knowledge thus acquired enabled us to proceed to the more complex problems relating to the degree to which combinations of two natural foods in various proportions supplement the deficiencies of each other.[6] In the present paper we present data showing the supplementary relations between the oat kernel and isolated food factors. The results may be briefly summarized as follows:

1. The oat kernel seems to contain proteins of a poorer quality than either the maize[5] or wheat kernel.[3] 9 per cent of oat proteins serve, when all the other dietary factors are properly adjusted, to induce a small amount of growth in the beginning of the experiment, but cessation of growth always follows after about a month and thereafter the animals remain stationary in weight or decline (Chart 6).

2. Casein does not appear to supplement the proteins of the oat kernel in a very satisfactory manner. The addition of 10 per cent of casein to 9 per cent of oat proteins, the other dietary factors being properly adjusted, does not induce growth at the maximum rate as do similar combinations of casein with wheat proteins[3] or with maize proteins.[5] Combinations of gelatin and oat proteins in about equal proportions have proven in our experience uniformly vastly superior to the similar casein and oat protein combinations.

3. The unidentified dietary factor fat-soluble A is present in very small amount in the oat kernel. It is not possible to supplement the oat kernel with inorganic salts and purified protein so as to induce growth beyond the 3rd month (Chart 3). The inclusion of butter fat or some other substance which supplies the unknown A prevents the failure at this point (Chart 5), just as it does in experiments where the ration consists of purified protein, salts, carbohydrate, and an extract which furnishes the dietary factor B.

4. The whole oat kernel, with the hulls removed in the laboratory by coarse grinding and fanning, will not induce any growth in young rats. A mixture of hulled oats or rolled oats with 5 per cent of butter fat induces very slow increase in body weight for at least 125 days (Chart 1, Lot 500).

[6] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

5. The oat kernel, like unpolished rice, wheat, wheat germ, maize kernel, alfalfa leaves, cabbage, and clover leaves, contains a liberal supply of the water-soluble B, the preparations of which induce relief from polyneuritis. This dietary factor we have found in abundance in all the natural foodstuffs in a fresh condition, so far as we have employed them in experimental work.

6. The addition of any single dietary component as protein, inorganic salts, or fat-soluble A does not supplement the oat kernel so as to induce appreciable growth (Charts 1 and 2).

7. The addition of two dietary factors to the oat kernel serves to induce good growth during the first 60 days when one of the additions is a suitable salt mixture. Without modifying the inorganic content of the ration when this is derived solely from the oat kernel, we have not seen rats make any marked increase in body weight. When the oat kernel is fed supplemented by but two dietary factors there is always early failure with loss of weight and death following the brief period of growth (Charts 3 and 4).

Failure has in our experience supervened earlier than when wheat or maize is fed with the addition of two purified food additions. The oat kernel, like the wheat kernel,[3] appears to cause injury to the animals when their diet is of such a character as to lower their vitality (Charts 2, 3, and 4). It is not necessary to assume the presence of something toxic in the oat kernel to account for the injury which results from the presence of a high content of oats in a monotonous food mixture taken over a considerable period. Oats produce feces of a pasty character which makes their elimination difficult, and in all probability tend to debilitate the animal.

This explanation becomes the more plausible when we consider the marked improvement of rats whose rations were identical except that the proteins of the oat were in one case supplemented by 10 per cent of casein and in the other by 9 per cent of gelatin. There can be no doubt that gelatin furnishes an amino-acid mixture which makes good the deficiencies of the oat proteins in a much greater degree than does casein (compare Charts 5, 8, and 9). The improved physiological condition of the animals which results from the superior quality of their protein mixture in the former case (Chart 9) may render them capable of over-

coming the difficulty of elimination of feces. If this is the real explanation, the stunting observed on the oat-casein combinations may be due to the absorption of the products of putrefaction from the intestine. Further experiments designed to test the validity of these assumptions are now under way, but several months must elapse before their records can be safely interpreted.

8. When the oat kernel is supplemented with casein, a suitable salt mixture, and butter fat, growth may proceed to the normal adult size at the normal rate in some animals, but in general growth is slower than the normal rate (Charts 5 and 7). We have been able to secure reproduction with these rations in but a single instance, and the young survived but 1 day.

Further investigations relating to the dietary properties of the oat kernel are in progress.

In all the experiments reported in this paper we have followed our usual practice of keeping from four to six rats together in a woven wire cage, the entire floor of which was covered by a pan of galvanized iron. Wood shavings were employed to keep the animals from contact with metal, and distilled water only was furnished. Iodine was supplied in the drinking water once each week in the form of iodine dissolved in potassium iodide. We also have fairly satisfactory records showing the quantitative food intake of the animals used in these experiments.

Details of the construction of the cages and of the devices used for feeding and furnishing water will be described elsewhere in the near future.

CHART 1. Lot 500 shows failure to grow and early decline in body weight of rats fed exclusively on hulled oats (Period 1) and slow but sustained growth over a period of 4 months on a mixture of hulled oats 95 and butter fat 5 per cent (Period 2). In Period 3 rolled oats were substituted for the hulled oats prepared in the laboratory, but this made no difference in the character of the curves.

Lot 501 shows the records of rats restricted to rolled oats as the sole food. Of the four animals in this group one was eaten by its companions after 4 weeks. The remaining three died at the end of the 6th and 7th weeks without having increased in body weight.

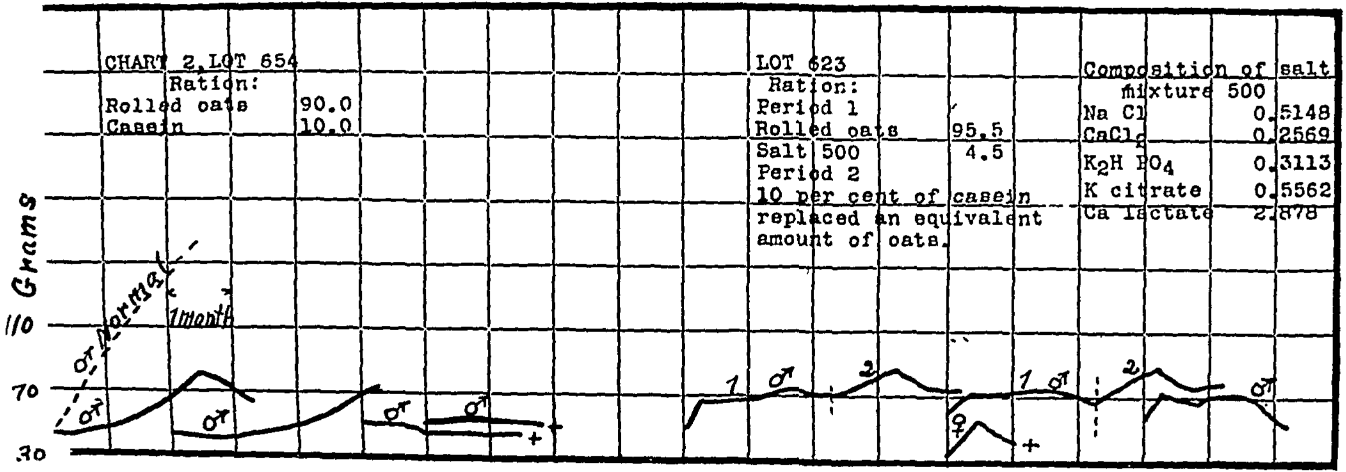

CHART 2. Lot 654 shows that young rats are unable to grow to any appreciable extent on the oat *kernel supplemented* with a pure protein. Two other dietary factors, the fat-soluble A, which is practically absent from oats, and the character and amount of the inorganic content must be taken into account in making good the deficiencies of the oat kernel.

Lot 623 illustrates the failure of rats to grow when restricted to rolled oats with inorganic salt additions (Period 1). The condition of the animals was such that the replacing of 10 per cent of oats by casein at the end of the 11th week did not cause growth.

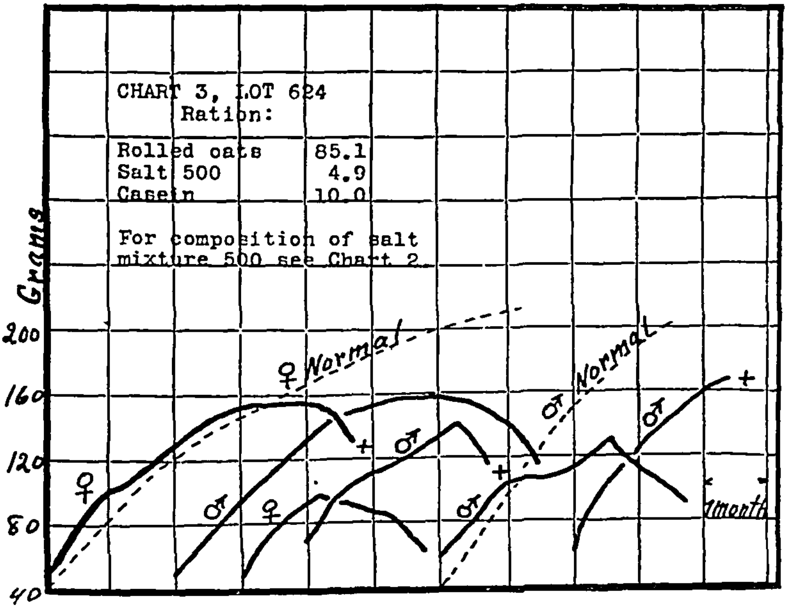

CHART 3. Lot 624 again emphasizes the marked tendency of rats to grow and apparently thrive for a brief period on rolled oats with two dietary additions; *viz.*, inorganic salts and purified casein. The rapid decline in this lot would have been entirely avoided by the inclusion in the diet of the dietary factor, the fat-soluble A, in the form of butter fat (see Chart 5). All dietary factors except the latter are contained in this food mixture and we interpret the cause of failure of this lot to the shortage of the fat-soluble A together with an injurious effect of the oat kernel on the animals.

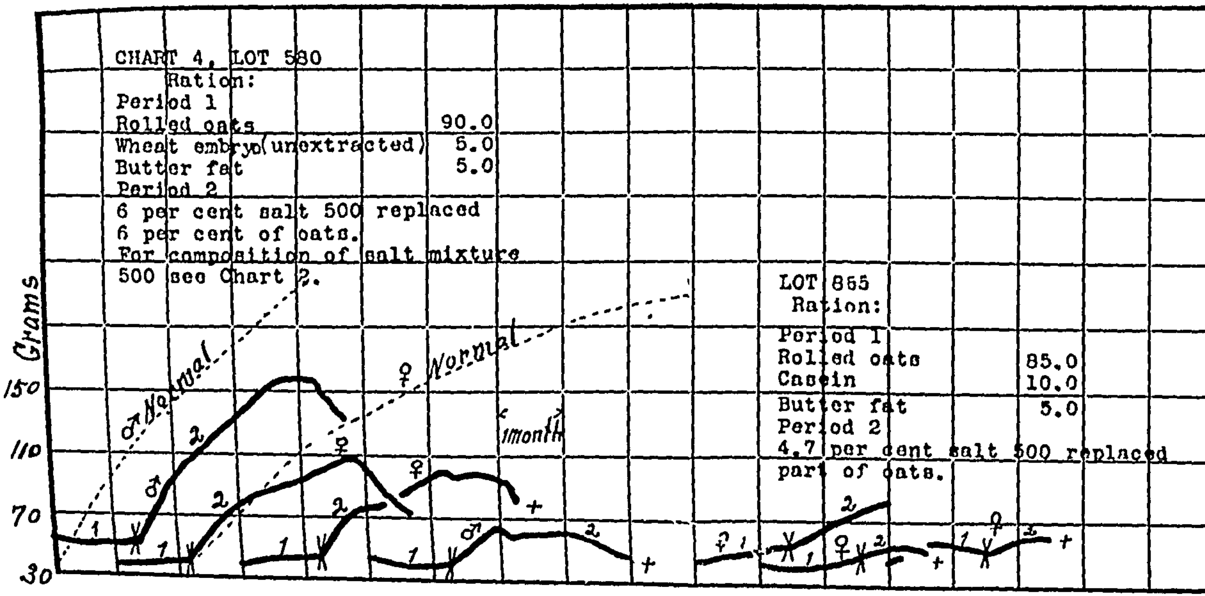
CHART 4, LOT 580
Ration:
Period 1
Rolled oats 90.0
Wheat embryo (unextracted) 5.0
Butter fat 5.0
Period 2
6 per cent salt 500 replaced
6 per cent of oats.
For composition of salt mixture
500 see Chart 2.
LOT 855
Ration:
Period 1
Rolled oats 85.0
Casein 10.0
Butter fat 5.0
Period 2
4.7 per cent salt 500 replaced
part of oats.
Grams
150
110
70
30
Normal
Normal
1 month

CHART 4.

CHART 4. Lot 580. This ration supplies rolled oats supplemented with the fat-soluble A in butter fat, and with an additional liberal amount of the water-soluble B in the 5 per cent of wheat embryo. In experiments with purified food mixtures 2 per cent of wheat embryo supplies enough of this factor to support growth at the normal rate.[1] 5 per cent of wheat embryo further supplies 1.5 per cent of protein of excellent quality.[4] The total protein content of the ration was 15 per cent. Even when thus supplemented rolled oats cannot support growth. After 6 weeks of growth suspension, the addition of 6 per cent of a salt mixture of suitable composition led to prompt growth at more than half the normal rate, but early failure ensued. We interpret this as supporting the evidence that the oat kernel when fed liberally causes injury to animals. Its effects are not markedly apparent when the diet is suitably adjusted as respects all its factors (Charts 5, 7, and 9) but serious injury may result when the ration is faulty for any reason, so that the vitality of the animal is lowered. In this group the vigor of the rats was impaired by the somewhat low content and poor quality of the proteins of the diet, and the debilitating effects of the oats became apparent. With a reduction in the oat content and an addition of 8.6 per cent of casein this diet is so modified that decline in early life is avoided (Chart 7). A similar diet containing oat proteins and gelatin is still more satisfactory (Chart 9).

Lot 855 illustrates the failure of rats to thrive on a diet of rolled oats, casein, and butter fat. When considered in connection with the other experiments reported in this paper it is evident that the character of the inorganic content of the ration, all of which except the phosphorus contained in the casein is furnished by the oat kernel, is the factor which is responsible for the results obtained with this food mixture (compare Charts 5, 7, and 9). The failure of these animals to respond with growth on the addition of a salt mixture after 6 weeks of stunting indicates lowered vitality.

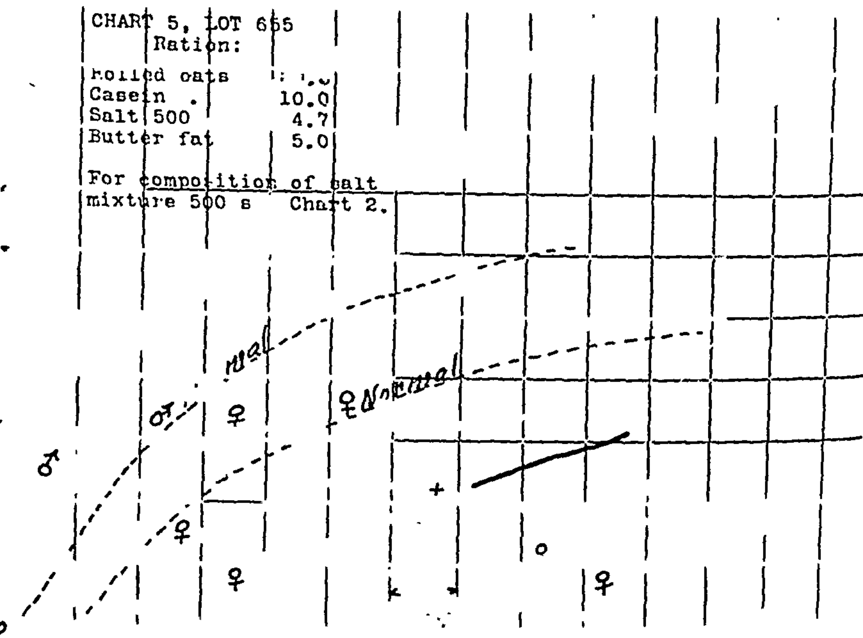

CHART 5. Lot 655 illustrates the ability of rats to grow with 80 per cent of rolled oats in their food mixture, when this is supplemented with casein, salts, and butter fat, and escape early decline and death. Without the salt mixture this diet does not support appreciable growth (Chart 4, Lot 855). Without the casein a brief period of growth is followed by decline and death (Chart 6, Lot 625). Without the butter fat (fat-soluble A) the same result is observed (Chart 3, Lot 624). Notwithstanding the presence of all essential chemical factors in this ration no reproduction was secured from these rats. This fact further supports the theory that oats exert an injurious effect when fed liberally over a prolonged period. It appears, however, from a comparison of Charts 5, 7, and 9 that casein and oat proteins do not form a protein mixture of as great value as do gelatin and oat proteins.

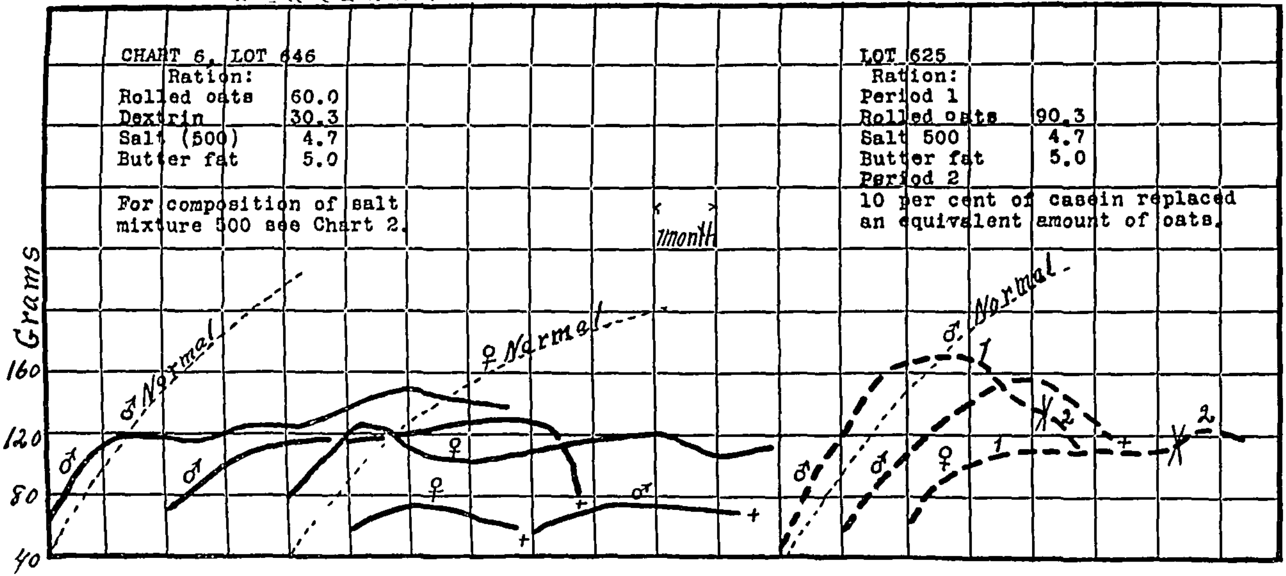

CHART 6. Lot 646 shows that 9 per cent of protein from rolled oats does not support growth beyond a limited extent. That the quality and quantity of the protein are the determining factors is made clear by comparison of these rats with those of Lot 655 (Chart 5) and with Lot 625 (Chart 6). The latter could grow but little on a similar diet containing about 13.5 per cent of oat protein, while Lot 655 (Chart 5), whose ration differed essentially from that of Lot 646 only in containing 10 per cent of casein replacing an equivalent amount of dextrin, were able in some cases to reach nearly the normal adult size.

Lot 625 illustrates the behavior of young rats given rolled oats with two additions, the fat-soluble A as butter fat and an inorganic salt addition. During 60 days growth was normal in two of the animals, but these suffered rapid decline during the 4th month. After being suspended in growth on this diet for a time the inclusion of casein in the diet did not induce recovery. The mixture of oats, salts, butter fat, and casein fed from the beginning would have promoted growth in many animals to the normal adult size without early failure (Chart 5). This ration furnished about 3.5 per cent of protein (Period 1) which is higher than is essential for the maintenance of growth[1, 7] when proteins are of good quality.

[7] McCollum and Davis, *J. Biol. Chem.*, 1915, xx, 415.

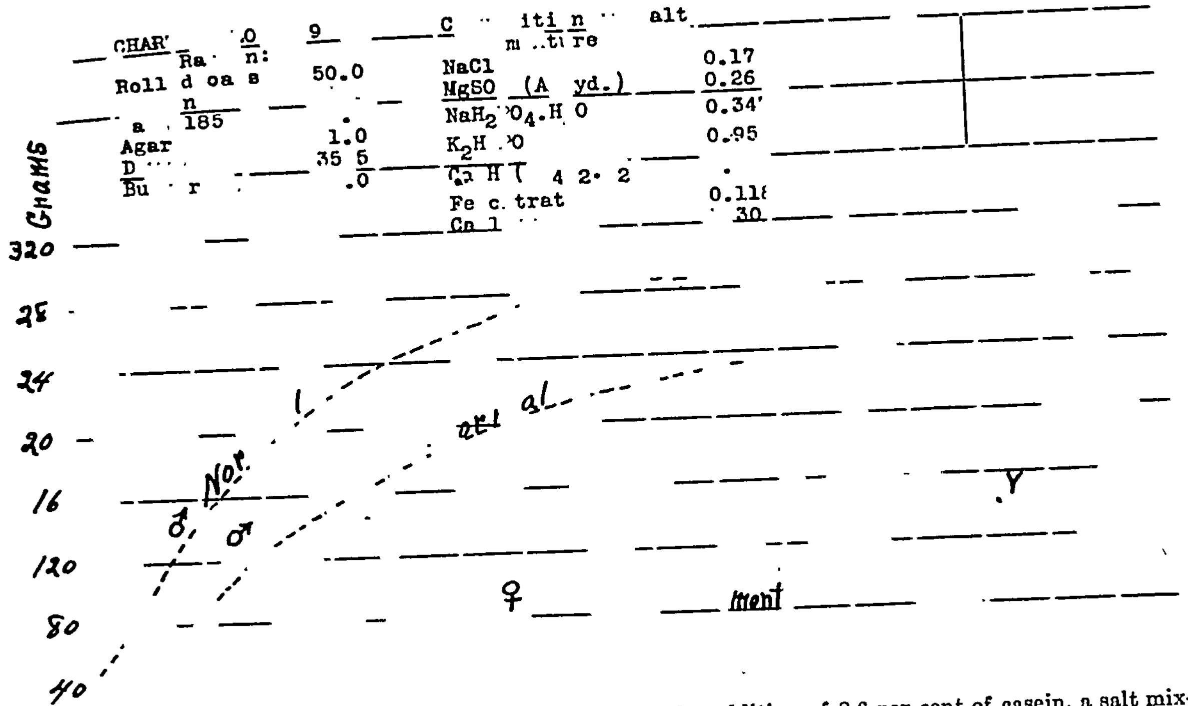

CHART 7. Lot 419 shows that it is not possible by the addition of 8.6 per cent of casein, a salt mixture, butter fat, and dextrin to 50 per cent of rolled oats, to induce the optimum of well-being in rats. Growth was not greatly depressed in the more vigorous animals, but two females during 11 months on this diet failed to produce any young, and a third produced at the age of 10 months a single litter which was immediately allowed to die. These animals were distinctly below the normal in vigor (compare

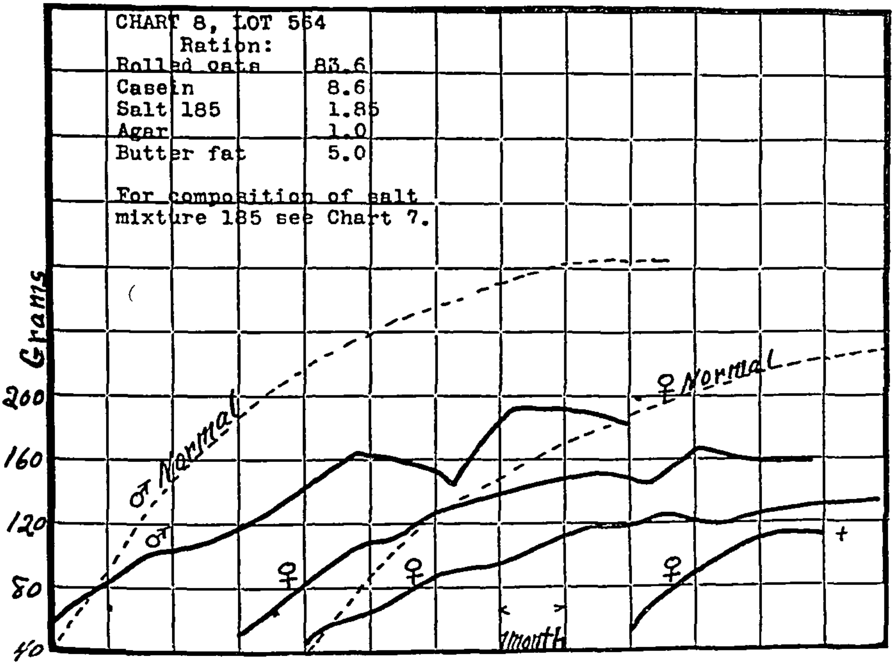

CHART 8. Lot 564 shows the records of rats whose ration was closely similar to that of Lot 419, Chart 7, except that the dextrin was replaced by oats. These rats were not quite so well nourished as were those receiving the lower oat content in the diet.

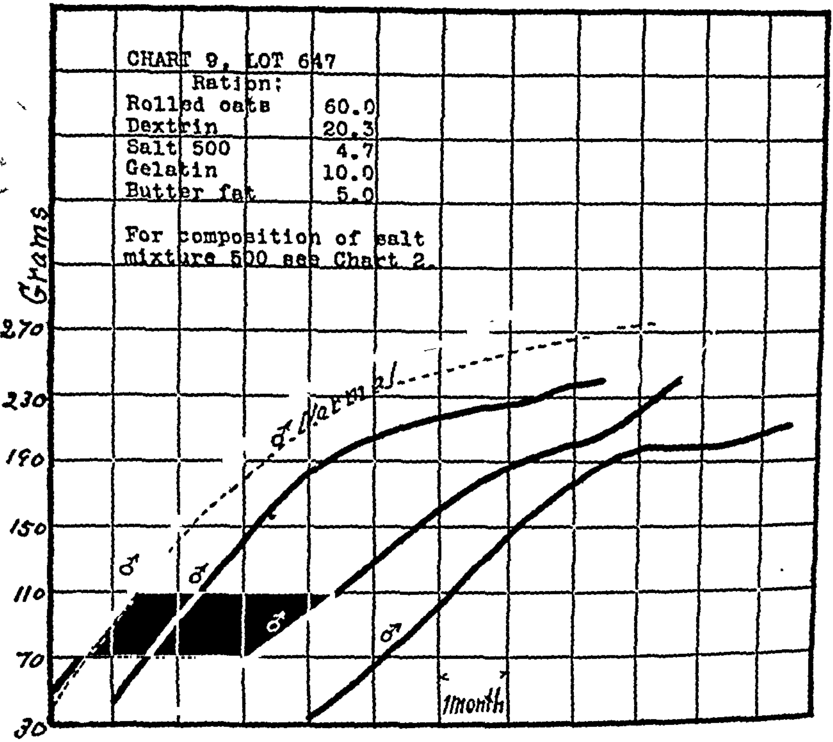

CHART 9. Lot 647 reveals the surprising fact that gelatin combined with oat proteins forms a decidedly better protein mixture than do casein and oat proteins (compare Charts 5 and 9). We have elsewhere shown that gelatin supplements well the proteins of the wheat kernel, but not at all those of the maize kernel.[8] Gelatin when taken as the sole source of protein or combined with maize is worthless. Properly combined with proteins of poor quality from certain sources it has a high biological value.

[8] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 483.

THE METHOD OF TIMED INTRAVENOUS INJECTIONS.

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PLATES 2 AND 3.

(Received for publication, February 1, 1917.)

INTRODUCTION.

If a soluble diffusible substance, such as a sugar, an amino-acid, or a salt, is made to enter the living organism at a constant rate for a long enough time, the tendency is toward the establishment of an equilibrium which is manifested by a constancy of the rate at which the organism rids itself of the substance as such, by excretion, or chemical change, or both. The rate of entry is then equalled by the rate of utilization plus the rate of elimination.

Since, during the maintenance of a uniform entry rate, the velocity of excretion cannot vary independently without affecting the quantity of substance left over in the body for utilization and *vice versa*, the rate of utilization cannot vary independently without affecting the quantity left for excretion, and since it is known that the rates of utilization and excretion are influenced by the amount of available substance, so the occurrence either of a constant rate of elimination or a constant rate of metabolism may be used as an index of equilibrium. Now if a substance of the character mentioned is made to enter the body of a higher animal or man at a constant rate, A, until equilibrium is established, observations may then be made on the rates at which various metabolic or other physiologic processes proceed during the period of equilibrium. If then under parallel conditions the same substance is introduced at a second rate, B, bearing to A some known numerical relationship, a second equilibrium may be established with a corresponding set of observations, and so on. In this way relatively exact quantitative data may be ob-

tained of a type which should favor the interpretation of more biological processes in terms of quantitative chemistry. We have in mind especially dissociation and the laws of chemical equilibrium. In a similar way we may compare the effects produced by bringing two different substances separately into the body at the same rate, or find the rates at which two or more substances have to be given in order to produce an identical arbitrarily selected effect.

Rates and Concentration.—To establish an equilibrium during the entrance of a given substance into a given animal at a given rate naturally implies the creation of some constant concentration of the substance in the body as a whole, *provided, however, the volume of the body is kept constant*—a condition which is not fulfilled, for instance, when in spite of the establishment of an equilibrium for one substance such as glucose, there is no equilibrium with respect to another such as water. What absolute concentration is actually produced must depend on many variables, such as the character and state of the substance, the species of animal, its physiological condition, the physico-chemical state of its tissues, etc. This absolute value could only be found by direct experiment for any specific case. To establish a constant concentration in the body as a whole would also imply, of course, a tendency toward the establishment of constant concentrations in each of the several tissues or body media corresponding to the relative solubilities of the substance in each, in accordance with laws which pertain to the partition of dissolved diffusoids among the phases of a heterogeneous system. The absolute values of these could also be found, if at all, only by direct observation in specific cases. *But for the purposes outlined above it is not necessary to know what concentrations are attained either in the body as a whole or in any of its parts,* although under conditions otherwise the same it might be assumed that they would vary in proportion to the rates at which the substances entered the body. In the case of simpler marine forms it is a common practice to control the rate at which an experimental substance enters the organism *by fixing the concentration of the substance in the circumambient liquid.* If the latter is changed frequently enough, or if its volume is made sufficiently great in proportion to the mass of the cells, then once the latter have ap-

proached equilibrium with the environment, molecules of the experimental substance migrate into the organism at a rate fixed by the concentration. With a gas, the concentration of which in the water is proportional to its volume percentage in the atmosphere over the water, the rate at which the substance enters the organism may be controlled by fixing its concentration in the air. And this method is available in the case of higher animals when the experimental substance is volatile like chloroform and ether, or a gas like oxygen or carbon dioxide, for the sea water then has its counterpart in the blood. The application of these principles in respiration studies by numerous workers has already led to notable achievements. *But in the case of higher animals and non-volatile substances regulation of the entry rate through the expedient merely of fixing a concentration is impractical, and recourse must be had to direct control of the entry rate into the blood, leaving the concentrations to adjust themselves.*

For well known reasons it is not possible to administer a substance by alimentary, subcutaneous, or other indirect routes and directly control the rates at which it will be taken up into the blood. On the other hand, if it is injected directly into a vein, the mechanical difficulty arises of sustaining the injection at a uniform rate for a long enough time. The problem of controlling the rate at which non-volatile substances are brought into the bodies of higher animals then resolves itself into one of mechanical apparatus.

Requirements for Apparatus.

A satisfactory apparatus should embody as many as possible of the following specifications. *In general*, it should be an instrument of precision, compact, portable, quiet in operation, for use by the bedside or in the laboratory. *The range of possible delivery rates* should be so wide as to meet the needs of experiments in laboratory animals and man. *The rate control* should be convenient and rapid, permitting of coarse and fine adjustments through a working range during the progress of a single experiment without interruption, and should allow a ready adaptation of the apparatus to the requirements of different experiments in which the ranges of rate differ widely. *An automatic rate-registering device* to indicate at every moment the actual delivery rate would be highly

desirable, and to this end the entire action of the mechanism should be positive so that the pump, when running at any particular speed shall inevitably move a corresponding volume of liquid. *The power and uniformity of action* should be such that a selected delivery rate may be sustained uniformly for 2 to 24 hours or more, with little attention in spite of variations of resistance such as may arise from changes of blood pressure or partial occlusions of tubing, cannulas, needles, or veins. *The accuracy* should be such that uncontrollable errors in the rate of delivery shall fall within the limits of error of methods available for detecting the physiological effects of such variations.

Parts coming into contact with the injection fluid should be quickly removable and replaceable without tools, for cleansing and sterilization. *The materials* should be selected with a consideration of their solubility in various injection media and their possible influence on the organism. Complex or delicate adjustments, valves, or other parts requiring undue care, or likely to become deranged or lost during ordinary use or cleaning, should be avoided.

Existing Types of Apparatus.

The various forms of apparatus described in the literature fall into three classes: (A) Devices in which pressure is imparted by gravity alone and the rate corrected by varying the size of the outlet. (B) Those in which pressure is imparted by a chamber of compressed air and the rate controlled as above. (C) Motor driven pumps.

It is unnecessary to consider in detail the disadvantageous features of apparatus of the first two types. The most notable use of a gravity device for prolonged intravenous injections was in the experiment of Henriques and Anderson,[1] who perfused a goat uninterruptedly for 20 days with a fair degree of accuracy. For a meter they used an ordinary dropping device inserted high up in the tube leading from the reservoir, a system which would not permit of a wide rate variation nor a change in the character of the liquid without recalibration. The principle of gravity flow, however refined and perfected, will necessarily involve

[1] Henriques, V., and Anderson, A. C., *Z. physiol. Chem.*, 1913, lxxxviii, 357.

cumbersome apparatus and considerable inconvenience, recommending itself only in special situations. Apparatus of Type B has been described by Kretschmer,[2] Rohde,[3] and others. Bulk, complexity, inconvenience, the undesirable solution of gases under pressure in the perfusion liquid, imperfect rate control, etc., are among their drawbacks. Motor driven pumps designed especially for organ perfusion work have been described by Brodie, Mandel, Friedmann, Straub, Kingsbury, and others. The problem of organ perfusion is not, however, identical with that of timed intravenous injections into the living animal. For perfusion purposes attention has been given especially to the injection of minute volumes of liquid in a pulsating stream, and especially by Kingsbury to materials and the form of the pulse wave. For the present problem, portability, adaptability to varying laboratory and bedside conditions, the convenient separation of parts for cleansing and sterilization, and above all positive, rapid, and exact regulation of rates through a wide range (*e.g.*, 10 to 2,000 cc. hourly), are features of prime importance. In the designs of perfusion pumps emphasis has not been laid on these points. On the other hand, the pulse form is of less significance for intravenous work.

Bock[4] was probably the first to use a motor driven pump for timed intravenous injections. His apparatus consisted of two glass syringes mounted side by side to form a duplex pump. The stems of the syringe plungers were screws and each passed through the center of a gear-wheel, as a bolt is screwed through a nut. When rotated, these wheels screwed the pistons in or out of the syringe barrels according to the directions of their rotation. The gear-wheels were turned in opposite directions by a single pinion engaging between them. Power was applied to the pinion by an electrical motor acting through a system of pulleys and belts. When either of the piston rods was screwed out to the end of a stroke it made an electrical contact, reversing the direction of the motor, and by the same device, a double three-way valve lying in front of the syringe barrels was operated by electromagnets. This complex apparatus delivered 0.2 to 10 cc. per

[2] Kretschmer, W., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 428.
[3] Rohde, E., *Z. biol. Tech. u. Methód.*, 1913, iii, 85.
[4] Bock, J., *Arch. exp. Path. u. Pharm.*, 1907, lvii, 177.

minute and was used in a study of renal function. The principle of reversing the motion of the pistons through the motor together with the belt transmission would necessarily involve irregularity and slippage. In 1913 Friedenthal and Willner demonstrated at the International Congress of Physiologists in Gröningen, a "*Praezisionsinjectorium*," concerning the details of which we have at present only the following program abstract: "The instrument serves the purpose of making possible the injection of specified solutions in the time desired and at the rate wished. It allows several experiments to be conducted simultaneously, or the injection of several solutions at different places into one animal. It permits further the preparation of unstable mixtures directly before injection. The instrument is also as appropriate for the most beautiful anatomic injections as it is for physiological and especially pharmacological and therapeutic injections." This is perhaps a later development of one of Friedmann's earlier perfusion pumps with multiple cylinders operated by a single machine, described in 1910,[5] an arrangement which would not be selected for the greatest exactness of rate control. In a report published in 1915[6] on the effects of prolonged and accurately timed intravenous injections of sugar are a cut and a brief description of a machine which has been used in this laboratory, consisting of a glass barrel and a metal piston driven by an electrical motor through worm and gear with a mechanically operated valve and a rheostat control. The model to be described presently represents merely a refinement of this machine. Raeder[7] has also mentioned using a motor driven Record syringe with a Bunsen valve to maintain even injections of salt solutions in a study of respiratory metabolism. The following cuts and descriptions are of an instrument built for us by William Gaertner and Company, of Chicago. Mr. Gaertner has embodied in it original solutions of some of the mechanical problems presented, such, for instance, as the link motion to meet the demand for a rapid means of adjusting the stroke of the pump, a feature which greatly increases the comfort of operation.

[5] Friedmann, E., *Biochem. Z.*, 1910, xxvii, 87.
[6] Woodyatt, R. T., Sansum, W. D., and Wilder, R. M., *J. Am. Med. Assn.*, 1915, lxv, 2067.
[7] Raedér, J. C., *Biochem. Z.*, 1915, lxix, 257.

New Apparatus.

The apparatus as a whole comprises two elements: (*a*) the pump and (*b*) the driving machine. Fig. 1 shows the pump unit taken apart for sterilization. Fig. 2 shows the same assembled and ready for insertion *en bloc* into the machine. Fig. 3 is a view of the machine from directly above showing the pump to one side of its bed on the driving mechanism in the position it would assume during detachment or replacement.

The Pump.—The pump (Figs. 1 and 2) consists of a metal piston with its rod, a ground glass cylinder, and a valve. On the extremity of the piston rod is seen the block by which the piston rod when in use is coupled to the carrier block of the driving machine (C in Fig. 3). The ground glass cylinder consists in this case of a 2 cc. Record syringe barrel with a special tip. The valve is seen to consist of a metal block perforated with a tapering hole and a tapering German silver core to fit. The principle of the valve, which is that of a three-way glass cock, is apparent from the cuts. In the center of the smaller end of the valve core is a screw projecting in the direction of its long axis. When the core is pushed into its place in the valve block, a spring washer is slipped over this screw and a circular milled nut screwed down to hold the block and core together with the proper tension. These details are visible in Fig. 1. On the lower end of the core, as seen in Fig. 2, is the slot to receive the valve lever by which the core is rocked to and fro, and the milled set nut to clamp the lever in place. It may be noted that the pump as a whole constitutes an interchangeable part which may vary in size and design and still be used on the same driving base. Thus, if it should be desired for special purposes to use the machine for timed injections of minute volumes of liquid, it would be possible to construct a special unit for this purpose. Since the stroke of the pump can be reduced literally to zero and the caliber of the barrel made as small as desired, the lower limit of the volume of liquid which can be pumped per minute depends wholly on the effectiveness of the valve.

The Driving Mechanism.—Power is provided by an electrical motor of $\frac{1}{20}$ horse power, and as it may be desired to use a single machine in different locations where the current available differs

in type, that shown in the cut (Fig. 3) is equipped with a universal motor which operates with any 110 to 120 volt current. For the best results, as with any motor, it is essential that the commutator be kept clean, and attention has been given to the details which make for the adequate protection and accessibility of this part. On the motor shaft in front of the motor casing is a heavy and carefully balanced leaden fly-wheel F, which adds tly to the momentum and steadiness of the machine when running; and further forward the shaft is coupled through a flexible connecting piece with a worm running in oil on ball bearings. Only the protective housing of the worm is visible in the cut at W. Engaging with the worm is a large horizontal gear, the center of which is visible at G, while the remainder is likewise encased for protection. The gear-wheel shaft bearings are provided for in a heavy pillar which forms a part of the base casting of the machine. The gear-wheel revolutions are to those of the motor and worm as 1 to 200 (subject to change). The motion of the gear-wheel is transmitted to the piston of the pump

It will be seen that in this machine the oscillations of the piston are directly proportional to the revolutions of the motor and cannot be otherwise. Also the valve is turned forcibly back and forth at the proper part of the stroke as an integral part of the motion of the whole machine. If the valve stops, the machine stops unless some part is broken or disconnected. Therefore, barring the factor of leakage in the valve and a wholly avoidable leakage about the piston or connections, the amount of liquid drawn into the pump and ejected during any interval is directly proportional to the number of strokes and this to the velocity of the motor. This would make it practical to provide the machine with a speed indicator for direct readings of the delivery rate at any moment, although this desirable factor has not been embodied in the present model. While the positive nature of the valve mechanism will naturally not insure against leakage due to imperfections in the valve itself, it has an advantage over delicate (ball, cone, or flap) valves in that the latter may be prevented from seating perfectly by gumming with thick liquids such as citrated blood, or from the lodgment of small particles between the valve surfaces. Leaks may thus develop during an experiment without affecting the speed of the machine and be detected only by timing the fall of the liquid in the supply burette. With a well made valve of the rotary type forcibly turned, this source of inconvenience is virtually eliminated and the machine becomes in reality a meter as well as a pump.

Performance.—The uniformity of the delivery rate with machines of the above design will depend practically on two factors: the perfection with which they are made, and the steadiness of the power current. An evenly balanced, smooth running motor, a true fly-wheel, well cut worm and gears, exact centerings and alignments, accurately fitted valves, proper oiling, etc., are of course essential. If these are satisfactory, the delivery rate is determined chiefly by the current and very little by variations of the pressure to be overcome in the tubing cannula, needle, or blood vessels. Two machines have now been subjected to heavy service in the wards and laboratory for a period of 12 months. One of them runs with great regularity and very quietly; the other is somewhat less quiet and requires more attention in order to secure even performances. They will aspirate liquids of vary-

ing viscosities such as water, citrated blood, or glycerol of 1.26 specific gravity, from burettes, flasks, beakers, etc., placed above or below them, and develop sufficient pressure to burst an ordinary rubber delivery tube if the latter is clamped while the machine is running, and this without materially slowing the rate of the machine. The stream emerging from an open delivery tube intermits twenty to forty times per minute according to the number of strokes. When the delivery tube terminates in a needle or cannula, the elasticity of the wall tends to equalize the pressure and an undulating stream results. The stream can be made continuous and uniform by the use of a section of thin-walled tubing with a narrow outlet or other equalizing device. For intravenous work it has proved satisfactory. A single machine has been run continuously for 19 hours by Sansum in actual animal experimentation, and they might be run for days. It has not been found difficult to make the quarter hour and hour discharge fall within 1 per cent of the desired values. With a favorable current and perfectly constructed machine, no attention whatsoever may be required for hours at a time to secure a uniform rate of delivery; but if the current fluctuates, or if the machine is imperfectly made or cared for, more attention will be necessary. By the rheostat alone the rate can be varied to a nicety between twenty and forty strokes to the minute with an ample working margin above and below. When the barrel of the pump has a bore of 8 to 9 mm., or about that of an ordinary 2 cc. all glass hypodermic syringe, the delivery rate may be varied by changing both stroke and speed from 1 cc. to 80 cc. per minute (60 to 4,800 cc. per hour). If higher rates of injection or aspiration were called for, which seems improbable, a larger barrel could be used, while the use of a barrel corresponding in size to a 0.5 cc. syringe permits of injection at rates as low as 0.2 cc. per minute (12 cc. hourly). The machine could be adapted readily for injections of smaller volumes per minute, but this has not been done with the present instruments. By the use of two (or more) machines with the discharging needle of one (or more) thrust into the delivery tube of another, or by the use of two or more machines injecting simultaneously into different sites in the same animal, a great variety of experimental combinations is made possible. We have, for example, injected a standard glucose solution at a

uniform rate with one machine, while with a second discharging into the delivery tube of the first, pure water, salt solutions, etc., have been superadded at specified rates. The power of aspirating fluids as well as injecting them, and to aspirate if desired at known rates, holds several possibilities.

There has been practically no inconvenience arising from occlusions of the needles, cannulas, and veins, except when the solution itself has been injurious to the vessel lining, even when injections have been made for long periods into small veins, such as the ear veins of a rabbit.

The present apparatus is doubtless subject to variation and further improvement. For special purposes, and where portability is not a factor, other mechanical solutions of the problem might be preferred, but the model described combines several qualifications of an ideal instrument and it has proved a valuable addition to the equipment of the ward and laboratory.[8]

EXPLANATION OF PLATES.

Plate 2.

Fig. 1. The pump unit taken apart, as for sterilization.

Fig. 2. The parts shown in Fig. 1 assembled and ready for insertion into the driving machine.

Plate 3.

Fig. 3. The machine from directly above, showing the pump to one side of its bed on the driving mechanism in the position it would assume during detachment or replacement.

F. Fly-Wheel.
W. Worm housing.
G. Exposed center of gear-wheel.
1, 2, 3, 4, 5. Links and levers which transmit power from gear-wheel to pump.
S. Movable sleeve on lever 2 for regulation of the length of the stroke.
C. Carrier block with post for fixation of the outer end of the pump piston.
G[1]. Guide on which the carrier block slides.
E. Eccentric arm for valve operation.
P. Insertion point for electrical plug.

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[8] Previous references to this pump have been made by Woodyatt, Harvey Lecture, 1916, and Wilder, R. M., and Sansum, W. D., *Arch. Int. Med.*, 1917, xix, 311. Later reports deal further with application of these principles and apparatus.

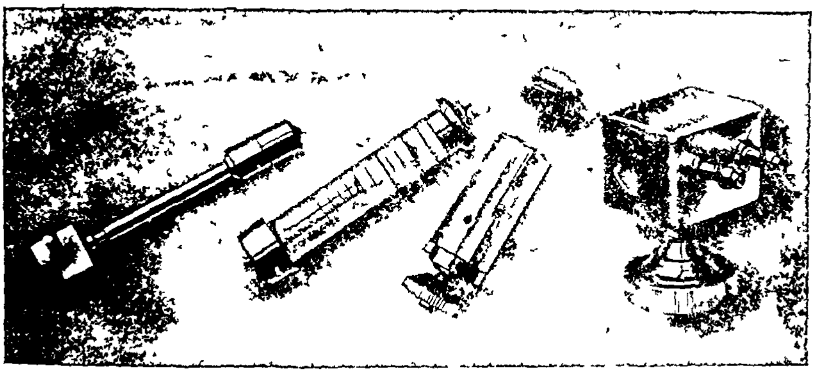

Fig. 1.

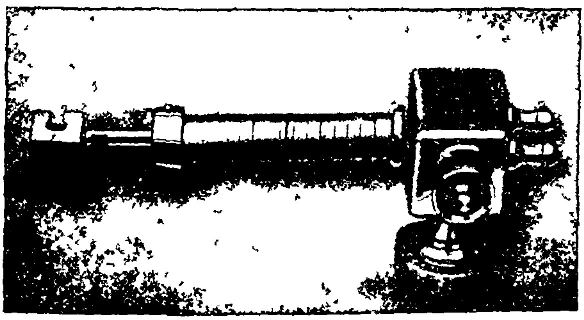

Fig. 2.

(Woodyatt Method of Injection.)

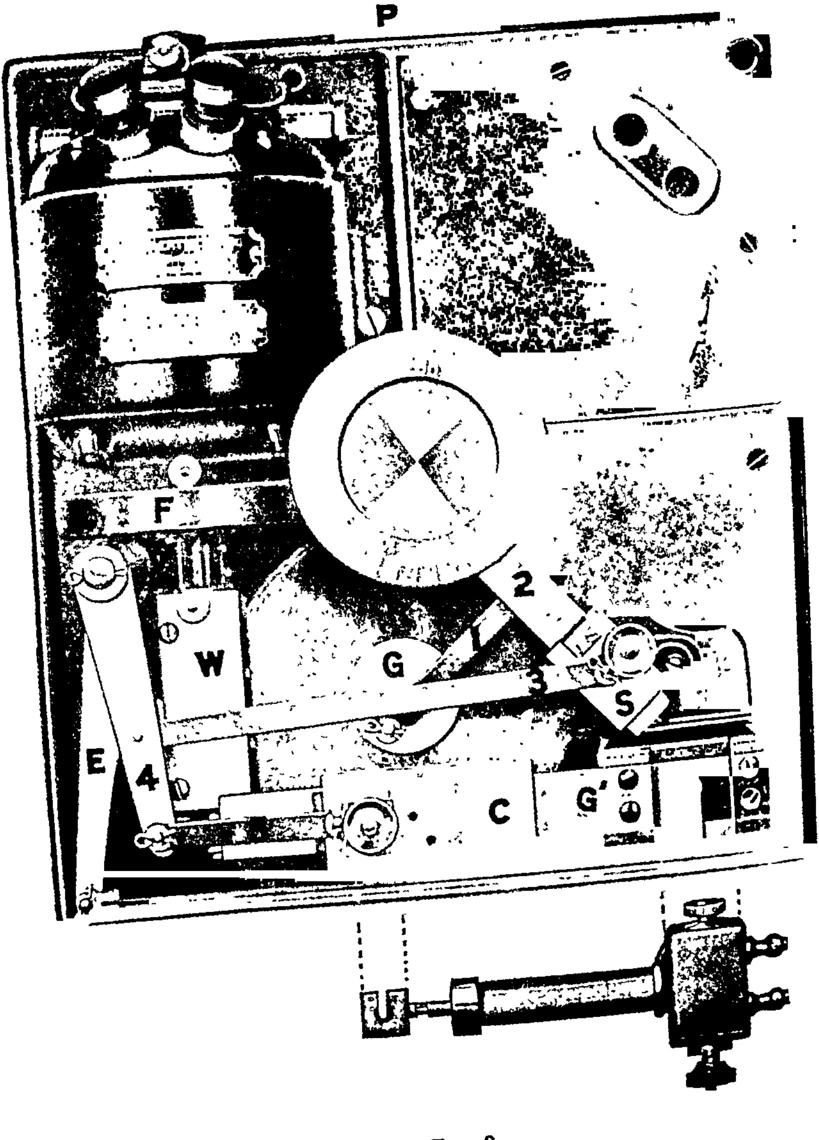

FIG. 3.

(Woodyatt: Method of Injection.)

THE EFFECT OF BILE AND BILE SALTS ON THE REACTION BETWEEN OLEIC ACID AND SODIUM BICARBONATE.*

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More than half of the fat ingested by an animal is absorbed from the small intestine as an aqueous solution of its fatty acids, or of its soaps, or of both, and this solution, of fatty acids in particular, is effected by the bile.

It has been shown by Strecker (1), Marcet (2), Latschinoff (3), and Kühne (4), and confirmed by Moore and Rockwood (5) that fatty acids are soluble in bile. It was shown by Moore and Parker (6) that oleic, palmitic, and stearic acids, and their sodium, calcium, and magnesium salts were practically insoluble in 5 per cent bile salt solutions unless these solutions contained 1 per cent of lecithin.

Pflüger (7), assuming that the alkali of the intestine, available for the neutralization of fatty acids, is sodium carbonate, attempted to show quantitatively the effect of bile on this neutralization. His method was to mix bile, sodium carbonate solution, and oleic acid or other fatty acids, the latter being present in amounts equivalent to the alkali, and after allowing the mixture to stand at 37° for several hours to extract the remaining free fatty acid by ether, and to dry and weigh it. In this way he found in one experiment that without bile the neutralization was 19 per cent of the oleic acid used, but in the presence of bile it was 10.57 per cent only. The presence of bile had decreased the ability of the two substances to react.

These low neutralization values which Pflüger found are easily explained by the observation of Chevreul that soaps hydrolyze in water solutions yielding free alkali and free fatty acid. This fatty acid from the hydrolyzed, preformed soap may be extracted by a fat solvent, and, therefore, in determining the amount of a fatty acid in a mixture containing

* The major part of this work was included in a thesis for the degree of Doctor of Philosophy, accepted by the Division of Medical Sciences of Harvard University.

soap it is necessary to prevent the hydrolysis of the soap. In a later investigation Pflüger (8) found that neutral oleate yielded 24.5 per cent of its combined fatty acid as free fatty acid when thoroughly extracted by ether, but did not apply this large correction to his earlier work or admit his error. Pflüger's experiments have been repeated, but with different results, as will be shown later.

Pflüger's assumption that the alkali of the small intestine available for the neutralization of fatty acids is sodium carbonate must be regarded as entirely without foundation in fact, as the following evidence shows.

Auerbach and Pick (9) measured the hydrogen ion concentration of the intestinal juice of dogs and found it to be 1×10^{-8} normal, and taking into consideration the disturbing effect of the small amount of carbon dioxide present, assumed that this was only a minimal value and that the true value is higher, 2×10^{-8} normal, which is little less than that of blood. These results are confirmed indirectly by the optimum hydrogen ion concentrations for trypsin, 2×10^{-8} normal, for pancreatic lipase, 1 to 2×10^{-8} normal, and for erepsin, 2×10^{-8} normal.

McClendon (10) found that the hydrogen ion concentration of the duodenal contents of normal men is 1 to 5×10^{-8} normal. These values indicate that the reaction of the small intestine is very faintly alkaline, corresponding to a dilute solution of sodium bicarbonate, perhaps containing a little free carbon dioxide, but not under any circumstances to a solution of sodium carbonate, no matter how dilute (11).

Although Pflüger's work in this field is given a prominent place in the text-books of physiological chemistry, a careful study of his papers shows that he started on a wrong assumption and that much of his experimental work was incorrect.

Since the alkali of the small intestine can be regarded as sodium bicarbonate, the extent of the soap formation during the digestion of fats will depend upon the ability of this alkali to neutralize fatty acids. It is the purpose of this paper to show by experiments *in vitro* that the presence of bile or bile salt solutions greatly accelerates this neutralization, with the formation of more soap (in some cases several times as much) than is formed in the same time and under comparable conditions when these substances are not present.

EXPERIMENTAL.

The problem was attacked in two ways, first by determining the amount of unused fatty acid (oleic acid was taken as a representative fatty acid) after it had been treated with sodium bicarbonate, and second by measuring the carbon dioxide given off in the reaction.

Materials.

Oleic acid was made from a high grade olive oil by saponification with alcoholic soda, setting free the fatty acids by the addition of dilute sulfuric acid, and subsequently separating the oleic acid by the lead soap procedure. The acid so obtained had an iodine number 96.9, and an acid number 196, as compared with 90.07 and 198, the iodine and acid numbers of the pure substance. It was nearly as colorless as water and was used in all the experiments unless otherwise specified.

Sodium oleate was made by exactly neutralizing oleic acid in alcohol solution with alcoholic soda, with phenolphthalein as indicator, and removing the alcohol by evaporation. The dry soap in absolute alcohol solution reacted neutral to phenolphthalein, 1 gm. of it requiring only one drop of 0.1 N sodium ethylate to make it distinctly alkaline.

First Method. Prevention of the Hydrolysis of Soap.

Solutions of sodium oleate in water were made up to the following concentrations: 0.1, 0.2, 0.3, 0.5, and 1.0 per cent. 100 cc. of these solutions were saturated by shaking with an excess of solid sodium chloride (40 gm.). The mixture was then extracted with 100 cc. of neutral chloroform in four portions. The filtered extract with the rinsings from the filter paper was titrated against 0.1 N sodium ethylate and the degree of hydrolysis determined. The results are shown in Table I.

TABLE I.

Free oleic acid in terms of soap.	Hydrolysis.	Soap in 100 cc. of solution.
gm.	*per cent*	*gm.*
0.0162	16.2	0.10
0.0192	9.6	0.20
0.0236	7.8	0.30
0.0280	5.6	0.50
0.0310	3.1	1.00

It is seen from Table I that, if care is taken to have the concentration of the soap as great as 1.0 per cent, the hydrolysis, as determined by this method, is a matter of only 3.1 per cent as

soap it is necessary to prevent the hydrolysis of the soap. In a later investigation Pflüger (8) found that neutral oleate yielded 24.5 per cent of its combined fatty acid as free fatty acid when thoroughly extracted by ether, but did not apply this large correction to his earlier work or admit his error. Pflüger's experiments have been repeated, but with different results, as will be shown later.

Pflüger's assumption that the alkali of the small intestine available for the neutralization of fatty acids is sodium carbonate must be regarded as entirely without foundation in fact, as the following evidence shows.

Auerbach and Pick (9) measured the hydrogen ion concentration of the intestinal juice of dogs and found it to be 1×10^{-8} normal, and taking into consideration the disturbing effect of the small amount of carbon dioxide present, assumed that this was only a minimal value and that the true value is higher, 2×10^{-8} normal, which is little less than that of blood. These results are confirmed indirectly by the optimum hydrogen ion concentrations for trypsin, 2×10^{-8} normal, for pancreatic lipase, 1 to 2×10^{-8} normal, and for erepsin, 2×10^{-8} normal.

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an excess of oleic acid from the preformed but hydrolyzed sodium oleate.

A comparison of Experiments 7 and 8 shows that the presence of bile makes the reaction with sodium bicarbonate more complete than is otherwise the case. That this cannot be accounted for by the alkali of the bile itself is shown by the low neutralization values obtained in Experiments 4 a, 4 b, and 6.

Experiments 7 and 8 were repeated using 20 cc. of a 10 per cent barium chloride solution. The use of this reagent entirely prevented the hydrolysis of the preformed soap, as shown by the fact that oleic acid could be quantitatively determined in the presence of sodium oleate. This was learned from several experiments. The chloroform extract was boiled to remove traces of carbon dioxide which are easily extracted from mixtures containing it and possess titratable values. The results are shown in Table III.

TABLE III.

No.	Bile.	Combined oleic acid.	Free oleic acid.
	cc.	*per cent*	*per cent*
7a	25	53.7	46.3
8a		27.6	72.4

Table III shows that the increase in the amount of oleic acid neutralized by sodium bicarbonate in the presence of bile over that without bile was 26.1 per cent. In Experiment 7 there was a similar increase of 24.0 per cent. The effect of bile is marked and is exactly the opposite from that found by Pflüger in a similar experiment with sodium carbonate, bile, and oleic acid.

Attempts to use this method of analysis with bile salts in place of bile failed because of the troublesome emulsions formed. The second method, that of determining the carbon dioxide production as an index of soap formation, was therefore resorted to.

Second Method.

Air was drawn through a tower (A) containing solid potassium hydroxide into a cylinder (B), 23 cm. high by 5 cm. internal diameter, having a slightly constricted opening fitted with a three

hole rubber stopper. The entrance tube from (A) reached to the bottom of the cylinder and had a perforated bulb at the lower end to decrease the size of the entering air bubbles. Nearly at the top of this tube, just under the stopper, was placed a rubber washer to prevent the froth from entering the exit tube. The stem of a small dropping funnel was inserted through the second hole. The third hole of the stopper contained the exit tube which was connected to a smaller cylinder (C) one-quarter full of concentrated sulfuric acid; and this in turn was attached to a calcium chloride U-tube (D). The calcium chloride in this tube had been saturated with carbon dioxide and the excess removed by washing with carbon dioxide-free air. The U-tube (D) was directly connected with a potash bulb of the Wetzlar type guarded from back suction of air and water by a large U-tube (F) containing solid potassium hydroxide and calcium chloride in alternate layers.

Air was drawn through the apparatus by connecting the U-tube (F) with a large aspirating bottle from which the water flowed by gravity into a large graduated cylinder. In order that the experiments might be made comparative, the rate of flow of the carbon dioxide-free air which ventilated the apparatus was made as uniform as possible in the several experiments.

The sodium bicarbonate solution was approximately 0.5 N and was standardized in the apparatus by adding an excess of 10 per cent sulfuric acid slowly from the dropping funnel in the cylinder (B) and drawing air through the apparatus until the weight of the potash bulb remained constant. 5 cc. of the bicarbonate solution were used in each experiment. 5 cc. of Merck's oleic acid, an amount in excess of the bicarbonate, were used.

Bile salt was prepared from ox bile according to the method of Tengström (12), and was that fraction obtained from the ferric chloride precipitate by decomposing it with sodium carbonate. The bile salt was completely separated from the contaminating sodium chloride and carbonate by repeated extractions with absolute alcohol. Analysis showed that the salt contained a small amount of sulfur and was therefore glycocholate slightly contaminated with taurocholate. A 2 per cent solution was used. This reacted neutral to litmus and phenolphthalein, but slightly alkaline to alizarin red. 10 cc. of the solution could be made

distinctly acid to this indicator by the addition of 0.05 cc. of 0.1 N hydrochloric acid.[1]

Certain bile salts were found to be soluble in oleic acid, as is also bile itself under certain conditions, and these solutions crystallize out on standing. In one experiment a solution of bile salt in oleic acid was used.

Shark bile salt, the fraction not precipitable by ferric chloride, and therefore corresponding in properties to the taurocholate of ox bile, was used in one experiment.

In two experiments lecithin and sodium oleate were substituted for bile salt.

Method of Procedure.

5 cc. of oleic acid were pipetted into the dry reaction cylinder (B) with 15 cc. of bile or bile salt solution and carbon dioxide-free air drawn through the apparatus with the potash bulb in place until all the carbon dioxide from the carbonates (if bile was used) had been removed. This preliminary procedure was found unnecessary with bile salt solutions.

The cylinder (B) was opened, 5 cc. of bicarbonate solution were quickly introduced by a pipette, and the cylinder was closed. A known volume of air was then drawn through the apparatus and the time recorded. The stop-cocks on the tube (D) were then closed, preventing the escape of carbon dioxide, the potash bulb was weighed as quickly as possible, and immediately put back in place. This operation was repeated until the weight of the potash bulb remained constant after two or three periods. All experiments were done at room temperature.

[1] On standing a short time a 2 per cent solution of sodium glycocholate becomes turbid and a flocculent precipitate falls out. If a fresh solution is immediately diluted with water to 0.2 per cent, it becomes milky and in a short time a small quantity of crystals forms. This is thought to be due to a hydrolysis of the salt with the formation of free bile acid, since the crystals were difficultly soluble in hot water and exhibited other characteristics of bile acid. A trace of alkali prevents this precipitation or crystallization.

PROTOCOL—*Concluded.*

Total time elapsed.	Total volume of aspirated air.	Total CO_2 evolved.
9. 15 cc. water. 5 cc. oleic acid. 0.2 gm. lecithin (Merck's). 5 cc. $NaHCO_3$ solution.		
min.	*cc.*	*mg.*
15	1,050	9
40	1,925	21
191	3,925	49
10. 15 cc. water. 5 cc. oleic acid containing dissolved bile salt. 5 cc. $NaHCO_3$ solution. Partial emulsion.		
15	1,000	52
35	1,850	90
100	3,050	95
168	4,650	102
198	5,250	103
228	6,100	104
268	7,100	104
11. 15 cc. 1 per cent Na oleate solution. 5 cc. oleic acid. 5 cc. $NaHCO_3$ solution. Partial emulsion.		
19	1,400	9
45	2,400	16
115	4,200	29
180	5,500	37

Explanation of Charts.

No. of experiment.			
1	10 cc. 10 per cent H_2SO_4,	5 cc. $NaHCO_3$ solution,	15 cc. water.
2	5 " oleic acid,	5 " " "	15 " "
3	5 " " "	5 " " "	15 " ox bile.
4	5 " " "	5 " " "	15 " 10 per cent ox bile.
5	5 " " "	5 " " "	15 cc. 5 per cent ox bile.
6	5 " " "	5 " " "	15 cc. 1 per cent ox bile.
7	5 " " "	5 " " "	15 cc. 2 per cent bile salt.
8	5 " " "	5 " " "	15 cc. shark bile salt.
9	5 " " "	5 " " "	15 cc. 1.3 per cent lecithin.
10	5 " " "	5 " " "	15 cc. water + bile salt in oleic acid.
11	5 " " "	5 " " "	15 cc. 1 per cent Na oleate.

Charts A, B, and C show the results of these experiments graphically. It will be noted that the curve of the velocity of the reaction between sulfuric acid and sodium bicarbonate under the experimental conditions is similar to that of oleic acid on sodium bicarbonate in the presence of undiluted bile, as shown by Curves 1 and 3.

A comparison of Curves 2, 3, and 7 shows how marked is the effect of bile or bile salt. A 2 per cent solution of sodium glycocholate is not so effective as bile which contains approximately 10 per cent of bile salts, as is to be expected. Curves 4 and 7 show that 10 per cent bile, in which the concentration of bile salt is approximately 1 per cent has the same effect as a 2 per cent solution of the bile salt itself. This added effect of bile is probably due to the presence of pseudo mucin, since Moore and Rockwood (5) found that the removal of this substance from bile rendered the latter practically incapable of dissolving fatty acids. Lecithin and sodium oleate increase the velocity of this reaction,

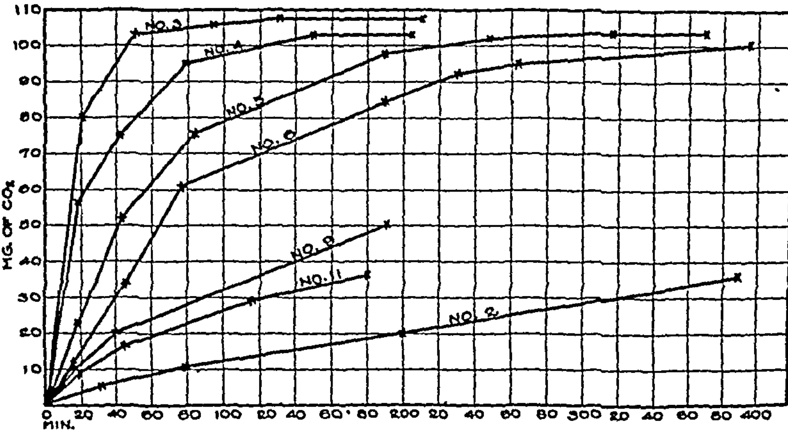


CHART A.

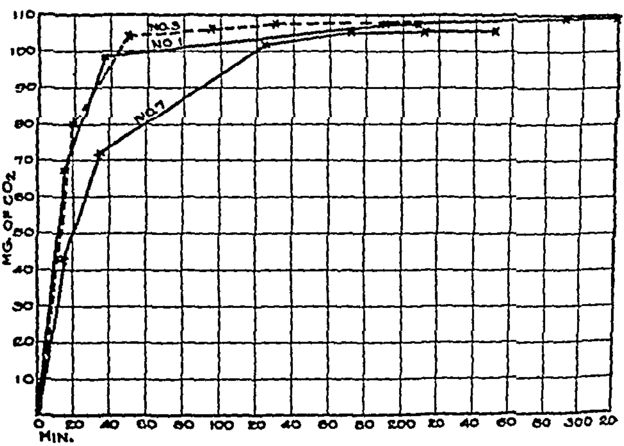
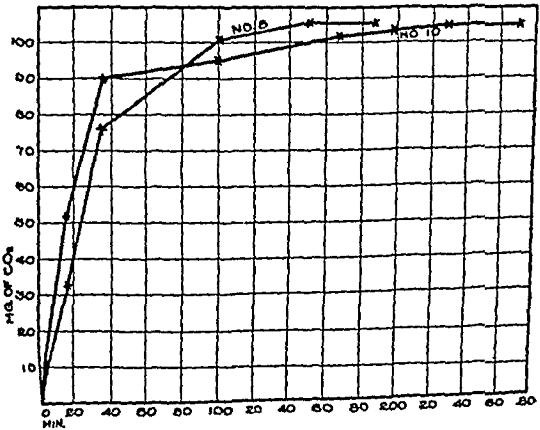


CHART B.



but are not so effective as 1 per cent bile, having a concentration in bile salt of approximately 0.10 per cent, shown by Curves 6, 9, and 11. Moore and Parker (6) found that bile salt itself had almost no solvent effect on fatty acids, but that if it contained 1 per cent of lecithin its solvent effect was comparable to that of bile. Shark bile salt has the same effect as ox bile salt, shown by Curve 8. Bile salt dissolved in oleic acid acts similarly to the same substance dissolved in water, shown by Curve 10.

The effect of bile is strikingly shown by a comparison of Experiments 2 and 3. In the former the soap formation corresponds to 48 mg. of carbon dioxide in 11 hours. In the latter, in the presence of bile, the soap formation corresponds to 80 mg. of carbon dioxide in 20 minutes.

CONCLUSION.

While it is dangerous to apply conclusions from simple experiments of this kind to the actual conditions in the small intestine during the digestion and absorption of fat, it seems reasonable to believe that the presence of bile makes possible a much greater soap formation from the fatty acids liberated during digestion than could otherwise be the case with an alkali as weak as sodium bicarbonate. A consideration of the physical properties of bile and bile salt solutions would lead one to expect the results obtained, but it seems of value to have demonstrated this experimentally. Attention is called to the fact that all of Pflüger's work in this field, published in several papers and cited in textbooks of physiological chemistry, is based on the erroneous assumption that the alkali of the small intestine, available for the neutralization of fatty acids, is sodium carbonate; and that much of his experimental work, based on this wrong assumption is, in his earlier papers at least, incorrect, because of an inadequate analytical procedure.

I am grateful to Professor Otto Folin, who suggested this problem, for many helpful suggestions and a constant interest in the work.

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VARIATIONS IN THE COMPOSITION OF HUMAN MILK DURING THE FIRST ELEVEN DAYS AFTER PARTURITION.

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By a study of the variations in the composition of human milk during the first few days of lactation it is possible to assist in an understanding of the metabolic changes taking place when the organism takes upon itself the function of food production as distinct from the elaboration of another individual. That this change in function must be accompanied by deep-seated metabolic changes is obvious. Hence if it is possible to trace regular variations in the most evident milk constituents, these variations may be traced to related processes in the mother.

Although innumerable analyses have been made to determine the per cent of protein, fat, and lactose in milk, there is a definite lack of information as to their interrelationship and variation in the same individual on several successive days.

The literature up to 1874 is thoroughly reviewed by Gorup-Besanez (1). The nearest approach to work of this nature is the report of Clemm (2) obtained from the study of one subject. Tidy (3) gives the average of analyses of composite samples taken during the first week after parturition. Camerer and Söldner (4, 5, 6), in a series of three papers, give the results of their analyses of fifty-seven samples of the composite milk from many women, and tabulate them with respect to the time after parturition. Meigs and Marsh (7), following the example of Tidy, report on the analyses of five composite samples taken between the 5th and 9th days after parturition. The information presented in these papers is of great value and serves as a standard by which other results of a similar general nature can be compared. They are, however, lacking in detail of advantage to the present problem.

Vernois and Becquerel (8), l'Heretiér (9), Kühn (10), Lehmann (11), Hinchcliff (12), Farrington (13), Penny (14), Eckles and Shaw (15), Eckles and Palmer (16), and others have demonstrated that in general the pro-

duction of cow's milk is influenced by various factors, such as breed, constitution, age, feed, stage of lactation, interval between milking, individuality, disease, etc. Notwithstanding this multiplicity of interfering factors the basic principles upon which milk production is accomplished are stable, and it is not illogical to suppose that a certain uniform variability in composition can occur during the progress of adjustment. It is the purpose of this report merely to point out these regularities in variation since sufficient data are not yet at hand to permit definite conclusions as to their cause.

The subjects of this investigation were healthy patients in the Los Angeles County Hospital. The diet was the same for all throughout the period of study. The milk production of eight women was studied.

Case.	Date. 1916	Case.	Date. 1916
1	Aug. 30–Sept. 7	5	Nov. 3–11
2	Oct. 6–14	6	" 3–11
3	" 17–25	7	" 5–13
4	" 17–25	8	" 5–13

The milk was collected by means of the breast pump at the same hour on the 3rd, 5th, 7th, 9th, and 11th days after parturition. Inasmuch as the patients are dismissed from the hospital the 12th day after delivery, milk collected thereafter would have been subjected to the influence of diet variation.

The total nitrogen, fat (lipoids), and lactose were determined in the fresh milk as sent to this laboratory immediately after collection.

The total nitrogen was estimated by the microchemical modification of Kjeldahl's digestion, devised by Folin and Farmer (17). The fat was determined by Meigs' (7) method. The lactose was determined in the protein-free filtrate by the use of Fehling's solution, with occasional polariscopic corroboration. Acetic acid, lead acetate, and heat were the protein precipitants used. All results are calculated to the basis of gm. of constituent in 100 gm. of milk.

Total Nitrogen.

In looking over the figures published by various investigators it seems as if the milk produced by the women of this country during the first 11 days of lactation is somewhat lower in total nitrogen content than is that of those abroad.

TABLE I.

A Comparison of the Averages from the Total Nitrogen Determinations in Milk of the 5th to the 11th Days after Parturition.

Total nitrogen.	Source.	Author.
per cent		
0.278	Average of the analyses of 13 composite samples.	Söldner (4). Camerer and Söldner. (5, 6).
0.244	Average of the analyses of 5 composite samples.	Meigs and Marsh (7).
0.255	Average of the analyses of 31 individual samples.	Hammett.

Further variations in nitrogen content are considered together with the variations in protein production.

The figures of Söldner and Camerer are taken from their third paper wherein they collect into one table the results of their whole series of analyses.

Protein.

Much work has been carried on in an attempt to differentiate between the various forms of nitrogen-containing substances in milk in order that a true picture of the protein output can be calculated. A discussion of this phase is beyond the scope of the present paper. While Camerer and Söldner (4, 5, 6) use the factor 6.25, and Eckles and Shaw (15) 6.38, Hawk (18) advocates the use of 6.37 as the figure by which to multiply the total nitrogen in milk in order to obtain the protein production. This last factor has been used in our calculations.

In Table II will be found the per cent of protein in the milk of eight women for the 3rd, 5th, 7th, 9th, and 11th days after parturition.

Group Variations for Each Day.—An inspection of the table will show that the limits within which the individual protein production may occur suffer a marked narrowing between the 3rd and the 5th days of lactation, the subsequent variation limits decreasing more gradually until on the 11th day the maximum variation from the mean is ± 18 per cent.

TABLE II.

The Per Cent of Protein and Total Nitrogen in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Case.	1		2		3		4		5	
Day.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.
3	1.99	0.312	5.79	0.908	4.81	0.755	2.06	0.322	5.09	0.800
5	1.62	0.255	1.97	0.310	1.90	0.298	1.58	0.248	2.32	0.364
7	1.49	0.235	1.83	0.287	1.65	0.260	1.60	0.252	1.92	0.301
9	1.91	0.307	1.77	0.277	1.70	0.267	1.55	0.244	1.82	0.286
11	1.64	0.258			1.59	0.250	1.41	0.221	1.38	0.216
Average	1.73	0.271	2.84	0.453	2.33	0.365	1.64	0.257	2.50	0.392

Case.	6		7		8		Average.		High.		Low.	
Day.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.	Protein.	Total N.
3	3.64	0.570	1.82	0.286	2.96	0.465	3.52	0.552	5.79	0.908	1.82	0.286
5	1.66	0.261	1.57	0.247	1.29	0.203	1.74	0.274	2.32	0.364	1.29	0.203
7	1.38	0.216	1.38	0.216	1.64	0.258	1.61	0.254	1.91	0.301	1.38	0.216
9	1.40	0.220	1.47	0.231	1.78	0.279	1.69	0.264	1.93	0.307	1.40	0.220
11	1 35	0.212	1.18	0.184	1.70	0.267	1.46	0.229	1.70	0.267	1.18	0.184
Average	1.88	0.295	1.49	0.233	1.87	0.293	2.00	0.314	2.73	0.428	1.41	0.222

Group Variations from Day to Day.—Coincident with the narrowing of the limits within which the protein production occurs there is a decline in the actual per cent of protein content of the milk, and it is during the period of most marked decrease in divergency that the most evident decrease in protein content takes place.

Individual Variations from Day to Day.—These show the same tendency towards a decrease with the progress of lactation.

Individual Variations from the Group Average for the Single Days.—From a qualitative point of view the individual variation from the group average for each day tends to be uniformly above or below that figure. This leads to the conclusion that the protein production plane is fixed for the individual, is independent of the plane of nutrition, and is dependent on the individuality, thus fundamentally correlating the findings of Eckles and Palmer (16) in their studies on the milk production of the cow.

Fat.

All reports agree that the most variable milk constituent is the fat. Hence it is not surprising that a comparison of the averages of various composite samples shows no consistent variations.

TABLE III.

A Comparison of the Averages from the Fat Determinations in Milk of the 5th to the 11th Days after Parturition.

Fat.	Author.
per cent	
3.18	Söldner, Camerer and Söldner (4, 5, 6).
2.45	Meigs and Marsh (7).
3.25	Hammett.

In Table IV there is a tabulation of the results of the fat determinations on the milk of the eight subjects studied.

TABLE IV.

The Per Cent of Fat in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Day.	1	2	3	4	5	6	7	8	Average.	High.	Low.
3	2.41	2.84	4.14	1.92	12.70	3.99	3.51	3.20	4.34	12.70	1.92
5	2.61	2.80	2.79	2.12	3.69	4.26	1.89	2.86	2.88	4.26	1.89
7	3.97	5.79	1.81	2.98	2.50	4.21	3.83	5.28	3.80	5.79	1.81
9	4.67	4.15	2.28	2.62	2.85	3.88	4.08	5.11	3.70	5.11	2.28
11	3.37		2.44	1.92	3.08	3.93	5.02	3.89	3.38	5.02	1.92
Average..	3.41	3.92	2.69	2.31	4.98	4.06	3.67	4.07	3.62	6.58	2.00

Group Variations for Each Day.—If we except the unusually high fat per cent found in the milk from Case 5 on the 3rd day there are no regularly occurring changes in the limits within which the fat per cent of human milk may be found during the early days of lactation. On the 11th day the maximum variation from the mean is ±44 per cent, a much greater divergency than that present in the protein production.

Group Variations from Day to Day.—From a quantitative standpoint the averages published by Camerer and Söldner (6)

and the results herein reported show an increase in the fat production after the 3rd day.

Individual Variations from Day to Day.—These show no tendency towards a diminution.

Individual Variations from the Group Average for the Single Days.—If we consider the direction of variation from the average, it is demonstrated that here as well as with the protein production there exists an individual tendency to uniformity of production plane. Inasmuch as the fat production is individually so extremely variable it is not surprising that the level of the production plane fails to maintain the same degree of consistency as is shown by the protein. Nevertheless the tendency is present and is of sufficient degree to afford still further evidence that the principle stated for protein production holds good in a like manner for the fat production of the lactating woman.

Lactose.

The average lactose content of human milk from the 5th to the 11th days after parturition according to various published reports is found in Table V. As far as these figures go they show that the American investigators find a higher sugar production during the early days of lactation than do those abroad. If the differences are valid they are significant of a higher lactose production plane in the subjects of the former workers.

TABLE V.

A Comparison of the Averages from the Lactose Determinations in Milk of the 5th to the 11th Days after Parturition.

Lactose.	Author.
per cent	
3.86	Gorup-Besanez (1), Clemm.
5.14	Gorup-Besanez (1), Tidy.
5.99	Camerer and Söldner (6).
6.53	Meigs and Marsh (7).
6.17	Hammett.

Table VI gives the results of the analyses for lactose of the samples of milk from the eight women studied.

TABLE VI.

The Per Cent of Lactose in Human Milk on the 3rd, 5th, 7th, 9th, and 11th Days after Parturition.

Day.	1		3	4	5	6	7	8	Average.	High.	Low.
3	6.49	6.20	4.63	6.51	2.31	5.72	6.11	5.44	5.43	6.49	2.31
5	6.44	4.15	6.56	6.81	4.96	6.52	6.86	6.33	6.08	6.86	4.15
7	6.71	5.72	6.56	5.95	5.85	6.49	6.67	5.70	6.21	6.71	5.70
9	5.76	6.11	6.81	5.91	5.91	6.86	6.78	6.44	6.32	6.86	5.76
11	6.58		6.81	6.73	5.37	6.81	6.58	6.04	6.42	6.81	5.37
Average..	6.40	5.55	6.27	6.38	4.88	6.48	6.60	5.99	6.09	6.74	4.66

Group Variations for Each Day.—The variations of the limits within which the lactose per cent is found show a fairly well marked tendency towards a narrowing. The maximum variation at the 11th day is only ± 6 per cent from the mean, a much lower value than that observed for either protein or fat.

Group Variations from Day to Day.—These figures like the results of Camerer and Söldner, show from the quantitative standpoint an increase in lactose production for the period. Underlying these group variations there must be metabolic variations of an important nature.

Individual Variations from Day to Day.—These gradually become less and less and are finally confined within rather narrow limits.

Individual Variations from the Group Average for the Single Days.—The variation of lactose per cent production shows for the individual the same qualitative uniformity of production plane as is shown by both protein and fat.

Variations in the Constituents.

In an extended study of the daily variations in the milk constituents of the cow Hinchcliff (12) found that an increase or decrease in fat content was usually accompanied by an opposite change in the per cent of sugar or protein.

In order to study the variations in the constituents of human milk I have calculated the per cent variation for each constituent from day to day and tabulated them in Tables VII, VIII, and IX.

TABLE VII.

The Per Cent Variation in Protein Per Cent from Day to Day.

Days after parturition	Case.							
	1	2	3	4	5	6	7	8
3– 5	−18.6	−65.9	−60.4	−23.3	−54.4	−54.4	−13.7	−43.4
5– 7	− 8.0	− 7.1	−13.2	1.2	−17.1	−16.9	−14.1	27.1
7– 9	17.8	3.3	3.0	− 3.1	− 5.2	1.5	6.5	8.5
9–11	−16.1		− 6.5	− 9.0	−24.2	− 3.6	−19.7	− 4.5

TABLE VIII.

The Per Cent Variation in Fat Per Cent from Day to Day.

Days after parturition.	Case.							
	1	2	3	4	5	6	7	8
3– 5	8.2	− 1.4	−32.6	10.4	−71.0	6.8	−46.2	−10.6
5– 7	52.1	106.8	−35.1	40.6	−32.3	−1.2	102.6	84.6
7– 9	78.9	−28.3	25.9	−12.1	14.0	−7.9	6 5	− 3.2
9–11	−27.8		7.0	−26.7	8.1	1.3	23.0	−23.9

TABLE IX.

The Per Cent Variation in Lactose Per Cent from Day to Day.

Days after parturition.	Case.							
	1	2	3	4	5	6	7	8
3– 5	− 0.8	−34.6	41.7	4.6	114.7	13.9	12.3	16.3
5– 7	4.2	37.7	*	−12.6	17.9	− 0.5	−2.8	−10.0
7– 9	−14.2	6.8	3.8	− 0.7	1.0	5.7	1.6	12.9
9–11	14.2	†	*	13.9	− 9.1	− 0.7	− 3.0	− 6.2

* No change.

† No result.

Arbitrarily throwing out all variations less than 3 per cent, it is seen that for human milk it can be stated that *during the first 11 days after parturition with an increase or decrease in lactose production there is a general tendency towards the opposite change in fat or protein production.*

The most marked reciprocal relationship exists between the fat and lactose. Underhill and Baumann (19), in a series of comparative analyses of blood sugar and blood fat, have found

a similar relationship to exist in the blood of dogs poisoned by hydrazine. They find that, "blood fat shows an increase to a maximum which is coincident with the condition of hypoglycemia induced by hydrazine." That such an interdependency should exist in such widely divergent cases is worthy of note, and is significant of close relationship of fat and carbohydrate metabolism.

SUMMARY.

In summing up the variations observed in the chemical composition of human milk during the first 11 days after parturition, it is necessary to consider them both from the quantitative and from the qualitative points of view.

From the quantitative point of view fat and lactose increase in amount during the period studied, while there is a falling off in the production of protein.

If we accept as an index of variability the individual variation from the group average, and the individual variation from day to day, the three constituents fall into the following order of decreasing variability: *fat, protein, lactose.* This order is changed to *fat, lactose, protein,* when we consider the qualitative aspect in regard to the group changes in variation limits and the group uniformity of change in production direction.

The protein mechanism is apparently the best regulated and is less dependent upon the factors controlling the fat and lactose production than they are on each other.

For the single constituents there is a production plane uniform for the individual subject. This plane of production is fixed for the individual, independent of the plane of nutrition and dependent on the individuality.

An increase or decrease in the lactose production is generally accompanied by a change in the opposite direction in the per cent of fat and protein.[1]

[1] To the Superintendent of the Los Angeles County Hospital are due my acknowledgments of his kindness in allowing me to obtain the material for this piece of work. It was through the efforts of Dr. Lyle G. McNeile that the collection of the samples was efficiently carried out.

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THE RATE OF UREA EXCRETION.

III. THE EFFECT OF CHANGES IN BLOOD UREA CONCENTRATION ON THE RATE OF UREA EXCRETION.

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On one side of the urea-secreting cells of the kidney there is blood and on the other side there is urine. The rate of urea excretion is the rate at which these cells transfer urea from blood to urine. Under these circumstances it seems probable that changes in the concentration of urea in the blood will have an effect on the rate of urea excretion.

If the concentration of urea in the blood were the only factor governing the rate of excretion, it would be possible to obtain a direct measure of its effect by determinations of the rate at different levels of blood concentration. But we have already shown[1] that the rate may vary when the blood concentration remains constant, and that there may be considerable differences in the rate, even when the concentration of urea in the urine as well as in the blood is constant. It is therefore certain that other factors besides the urea concentration of the fluids on either side of the kidney cells exercise a determining influence on the rate of excretion. And unless these other factors can be kept constant, any observed rate of excretion may be higher or lower than that which would have resulted had the blood concentration been the only operative factor.

We have not succeeded in framing experimental conditions under which we could be sure that this necessary constancy was attained. This of course was to be expected, since some factors which seem likely to influence the rate of work of the kidney

[1] Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1916, xxiv, 203.

cells, as, for instance, the rate of flow of blood, could not be directly controlled in our experiments. But we have found that under certain conditions these unknown and uncontrolled factors tend to increase the rate of urea excretion above that usually found at the observed blood concentration, while under other conditions the rate tends to be decreased. Since these factors alter the rate in both directions, an average of a number of observations at different levels of blood concentration will give an approximation to the effect of blood concentration alone. The accuracy of this approximation will depend on the degree to which these observations represent an equal distribution between instances where the rate has been increased under the influence of other factors than blood concentration, and those in which it has been decreased.

The general method we have followed was to estimate the average blood urea concentration for intervals of 15 to 120 minutes during which the urine was collected. The rates of urea excretion were expressed as gm. of urea per hour. Points were plotted on a scale, so that the ordinates were equal to these rates, and the abscissæ to the corresponding measures of blood urea concentration. The curve drawn through these points will measure the effect of changes in blood urea concentration on the rate of urea excretion more or less accurately according as the above condition has been fulfilled.

The subjects were adults, at or below 35 years of age, who were free from any sign of kidney disease.

The higher levels of blood urea concentration were obtained by the administration of urea in doses of 20, 30, or 40 gm. of urea, the intermediate by observations taken before or after the concentration had reached its maximum following urea ingestion, while a considerable variety of lower concentrations was found in subjects who had taken no urea.

The conditions as regards food and water intake were very variable. In some experiments no food was taken for as long as 20 hours, in others considerable quantities of urea-forming food had been consumed. The water intake varied from no water for 12 hours or more before an experiment to quantities of 1,500 cc. before, and large quantities during the period of observation. No experiments in which drugs were given are included.

Collections of urine were made over periods which were made as short as was compatible with accuracy, and the average blood urea concentration for each of these periods was calculated. The error in the actual estimation of urea in the blood is small in comparison with the possible error which might arise in calculating an average concentration over a period of time from the concentration found during a few moments of that time. For the blood urea concentration is constantly changing, and it does not follow that the concentration found at the middle of a half hour period accurately represents the average concentration throughout the whole of that time. As a rule, however, changes in urea concentration rise or fall fairly evenly over periods of hours. The majority of our observations were made in consecutive series extending over 3 to 12 hours, and the blood urea was determined every hour or every half hour. In this way a curve of urea concentration was obtained and from this the average concentration for each period was calculated. This was particularly useful in those experiments in which urea was administered, for there the concentration rose and fell from a maximum. Where there was a smooth curve of this sort there was probably little error in the calculation of the average concentration for each period, except for that period in which the maximum was attained.

There is still another possible source of error in connection with the determination of the blood urea concentration. In experiments of the type we are concerned with, it seems to have been tacitly assumed that the concentration of urea found in blood removed from an arm vein is the same as the concentration which exists in the blood reaching the urea-secreting cells of the kidney. But since we know that urea is not secreted by the glomeruli, and have reason to conclude that a large part of the water of the urine is, it seems likely that the concentration of the blood which leaves the glomeruli is higher than that of the systemic blood in proportion to the amount of water abstracted from it in the glomeruli. In experiments in which the volume of urine is very large, it is conceivable that the difference thus produced might lead to appreciable error.

The estimation of urea in the urine was done in duplicate. As in the case of the blood urea, the greatest source of error is

probably not the technical one, which can be measured, but one arising from the conditions under which the experiments were conducted. Especially, we believe, in cases in which the urine volume was small, considerable error may sometimes have resulted from incomplete emptying of the bladder. The subjects, however, were asked to take special pains to make urination complete, and where the urine volumes were small, the periods over which the rate of excretion was determined were lengthened. In such cases more than one estimation of blood urea concentration was sometimes made for each period.

Part of the data has been given in tabular form in a previous paper,[1] though a considerable number of observations made since that time have been added.

All the observations made under these varying physiological conditions have been plotted in Fig. 1. The general trend reveals the existence of a relationship between the concentration of urea in the blood and the rate of urea excretion. The curve shows the theoretical quantitative effect of any given degree of change in blood concentration, other factors remaining constant. The degree of scattering indicates to what extent other factors than blood concentration may modify the rate of excretion.[2]

There are certain defects in the data which have to be considered as probably detracting from the accuracy of the curve. Of these, the sources of error we have described above in the estimation of the rate of excretion and of the blood urea concentration cannot be considered as important in relation to the extent of the scattering seen in the graph. Appreciable errors were certainly only occasional, whereas the scattering is general. The most important and probable cause of a deflection of the curve from its true course would be a want of balance between instances of acceleration and of depression of the rate of excretion due to other causes than changes in blood concentration. The drinking of large quantities of water tends to accelerate the rate of excretion, and abstention from water to decrease it, apart from any alteration in blood concentration. There is a fairly even distribution of observations made under

[2] The curves were drawn for us by Mr. Otis, of Stanford University, by means of a method he has described which is called the "method of rank correspondence." Otis, A. S., *School and Society*, 1916, iv, 716, 750.

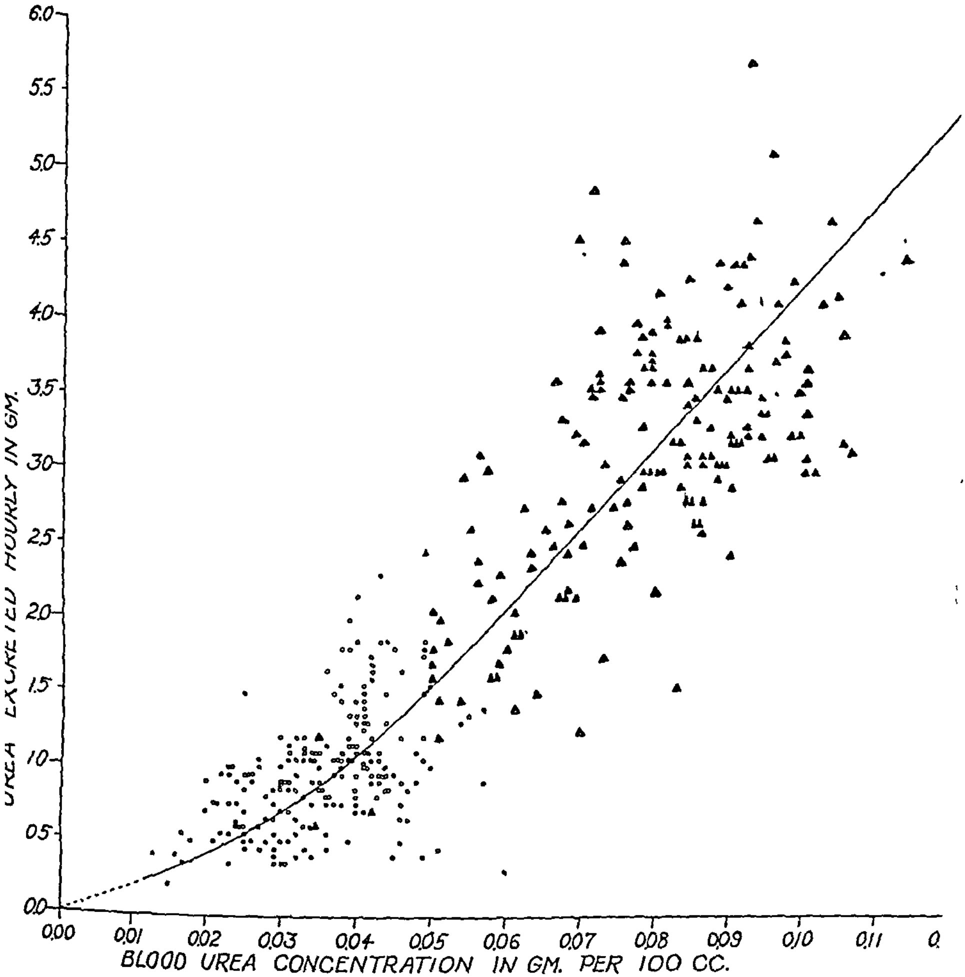

FIG. 1. The effect of changes in blood urea concentration on the rate of urea excretion. Observa- tions on a group of individuals. The circles indicate experiments in which no urea was given, and th triangles those in which urea was administered.

these two conditions in the subjects who took no urea, but in those experiments in which urea was administered, there are too many taken from water drinking experiments. This may account in part for the slight alteration in the direction of the curve at the higher levels of blood urea concentration as opposed to the lower. Yet even if such experiments are excluded, there is a definite tendency after the administration of urea for the rate of excretion to be greater than would be expected from observation of the result of alteration in blood concentration in experiments in which no urea was given. It has been noted from observation of the renal vessels in animals that urea injections may be followed by an apparent increase in the rate of flow of blood through the kidneys. It may be that part of the acceleration of the rate of excretion in man after the ingestion of urea is due to this cause, as well as to the increased urea concentration of the blood.

Although the absolute differences between the rates of excretion observed at each level of blood concentration increase as the concentration rises, yet relatively they become steadily smaller. Thus at 0.03 per cent the probable range of variation is over 200 per cent of the average, whereas at 0.09 per cent it is only about 75 per cent. Since it is the relative and not the absolute differences which measure the degree of variability in kidney function, we may conclude that the urea-excreting function of the kidneys tends to become more uniform the greater the urea concentration of the blood. When there is only a small amount of work to do there are great differences in the rate of work, but when the load is increased, the output becomes less variable.

The great variability in the rates of excretion at every grade of blood concentration is of particular interest in relation to the high degree of uniformity in the 8 to 24 hour rates at which subjects excrete urea added to a constant diet.[3] The differences in these experiments were no greater than those which might have resulted from individual fluctuations in the rate of protein catabolism. This uniformity cannot be ascribed only to the equality of conditions as regards food and water intake, for we have recorded instances[1] in these subjects where the rates of

[3] Addis and Watanabe, *J. Biol. Chem.*, 1916, xxvii, 249. There was no egg given with the noonday meal, as is incorrectly stated in this paper.

excretion during short periods of time showed a variation not markedly lower than that shown in Fig. 1, even when the blood concentration was constant. We believe that the uniformity arose mainly from the fact that the rate was observed over long periods of time (8 to 24 hours), while in the experiments given in this paper the time of observation was relatively short (15 to 120 minutes). Though kidneys which are called on to perform equal amounts of work may vary their rate of work widely from hour to hour, their total performance for the day is relatively uniform. If this is the true explanation, we may conclude that those unknown factors which cause the kidney to alter its rate of work have only a temporary and evanescent effect, and that a depression of kidney activity during 1 hour of the day is counterbalanced by a later acceleration, so that the total output remains the same.

The fact that in twenty-nine young adults a high grade of uniformity was found in the 8 to 24 hour rate of *excretion of* administered urea[3] indicates that there were no fixed individual peculiarities in the kidneys of that group. The subjects of the present observations include all of the above group, and the additional subjects were similar in their age and freedom from evidence of kidney disease. It seems therefore improbable that the scattering shown in Fig. 1 can be due to permanent individual differences in kidney structure. But it is possible to give direct evidence in regard to this point from our data. A considerable number of observations (110) were made on one subject under all the various experimental alterations in food and water intake. Fig. 2 shows the curve and the degree of scattering in his case. Since the kidneys of this individual show as great a variability as is manifested by the kidneys of all the other subjects, we may conclude that permanent anatomical differences are not responsible for the variations in the rate of urea excretion independent of changes in blood concentration during short time intervals. The causes of these variations must be sought for among temporary, irregular, and counterbalancing alterations in the environment of the kidney.

In the next paper evidence is presented showing that alterations in the urea concentration of the urine play no appreciable part in the production of these variations.

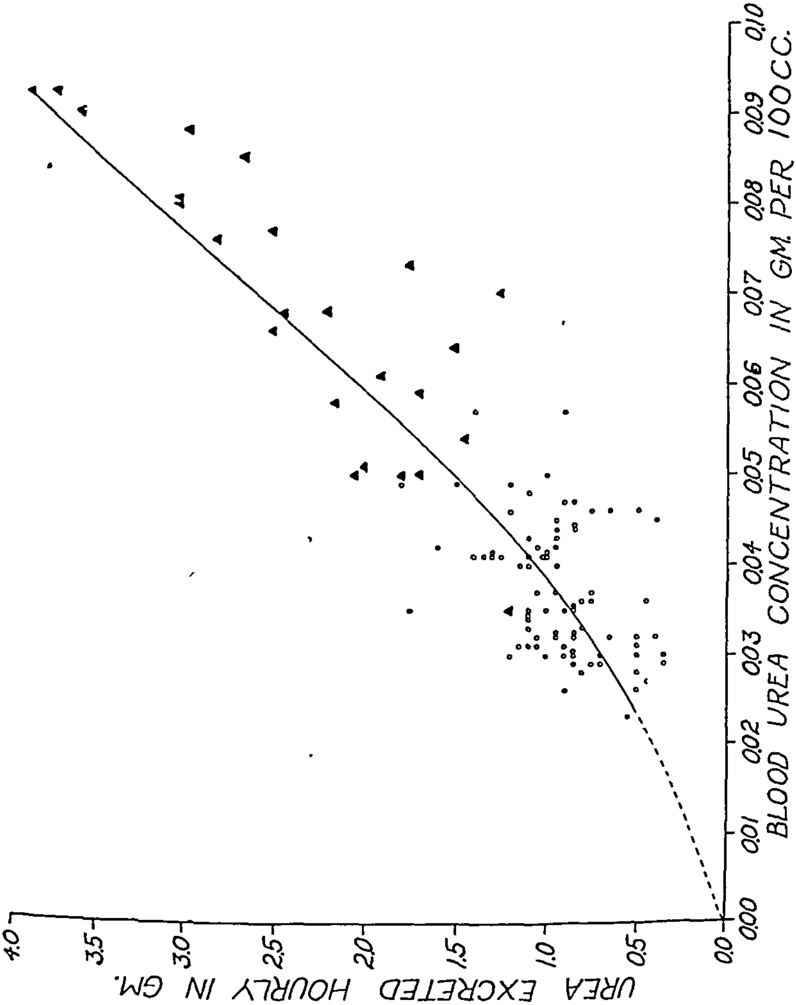

Fig. 2. The effect of changes in blood urea concentration on the rate of urea excretion. Observations on a single individual.

CONCLUSIONS.

1. A curve has been constructed which indicates the effect of changes in blood urea concentration on the rate of urea excretion in man.

2. The wide scattering of the observations from which the curve was constructed shows that other factors than blood urea have a pronounced effect on the rate of urea excretion.

3. There is a relative decrease in the degree of scattering, as the blood urea concentration increases. This is interpreted as indicating that the greater the stimulus to increased work in excreting urea, the less subject the kidney becomes to influences tending towards variability in its rate of work.

4. The variability in the rate of urea excretion at every level of blood urea concentration during the short periods of time chosen in these experiments is in marked contrast to the uniformity in the rate of excretion of administered urea over periods of 8 to 24 hours. This is taken as indicating that unknown factors lead to short-lived variations in the rate of excretion, and that these variations tend to counterbalance one another over longer periods.

5. The degree of scattering in one individual is as great as the scattering in the whole group. This makes untenable the hypothesis that permanent individual peculiarities, such as might arise from anatomical differences in kidney structure, are responsible for the variability in the rate of urea excretion revealed by the scattering shown in Fig. 1 for the group.

THE RATE OF UREA EXCRETION.

IV. THE EFFECT OF CHANGES IN THE VOLUME OF URINE ON THE RATE OF UREA EXCRETION.

By T. ADDIS and C. K. WATANABE.

(*From the Laboratory of the Medical Division of Stanford University Medical School, San Francisco.*)

(Received for publication, January 22, 1917.)

Since the concentration of urea in the blood influences the rate at which urea passes through the urea-secreting cells of the kidney, it seems likely that the concentration of urea in the urine in contact with the internal surfaces of these cells should also have an effect. A high concentration would tend to delay, and a low concentration to facilitate the discharge of urea from the cells, at least if the mechanism of this discharge depended on a process of diffusion. It is, of course, obvious that we cannot determine the actual urea concentration of the urine which lies next to the urea-secreting cells. The anatomical evidence given by Leschke,[1] which has been confirmed by Oliver,[2] demonstrates that urea is secreted by the cells of the proximal convoluted tubules. Since there is every reason to believe that a great part of the water of the urine passes through the glomeruli, we should expect that a series of different concentrations, increasing in proportion to the distance from the glomerulus, will be present along the reaches of each proximal tubule. And when, further, the possible occurrence of secretion and absorption of water at lower levels of the tubular system is remembered, it becomes apparent that we cannot assume any close or constant relationship between the urea concentration in urine collected from the bladder, and the urine as it lies in the proximal tubules. We have grounds, however, for believing that a rough general parallelism exists, and that when the urea concentration of collected urine is found to be greatly

[1] Leschke, E., *Z. klin. Med.*, 1914, lxxxi, 14.

[2] Oliver, J., *J. Exp. Med.*, 1916, xxiii, 301.

decreased as the result of an increase in volume produced by water drinking, there will also be a decrease in the average concentration in the proximal tubules.

In observations on the volume of urine of subjects under constant dietary conditions,[3] we found that the urine volume was increased when the rate of urea excretion was approximately doubled by the administration of urea. On the other hand, the rate of urea excretion was not increased when the urine volume was doubled by the administration of water. This raises the question as to whether there may not be a general relationship between rate of urea excretion and volume of urine of such a nature that the urea excretion influences the volume, in contradistinction to the idea of the volume influencing the urea, which is implied in the above mentioned theoretical considerations as to the possible effect of the urea concentration of the urine.

With the data at our disposal, we can determine whether there is evidence of any general relationship between the volume of urine and the rate of urea excretion, such as would lend support to either of these hypotheses. This might be accomplished by determining the net correlation between these two factors by means of a formula for partial correlation. But it was pointed out to us that the distribution in our material of the three variables (rate of urea excretion, volume of urine, and concentration of urea in the blood) was so far from normal as to render any coefficient of correlation somewhat misleading. It was therefore considered better to eliminate the factor of blood urea concentration by plotting the rates of urea excretion as plus or minus differences between the observed hourly rates and those which the curve shown in Fig. 1 of the preceding paper indicated as normal for the blood concentration found in each instance.

These differences are represented in Fig. 1 by the ordinate, while the abscissa measures the volume of urine per hour. It is apparent that there is no general relationship between the volume of urine and the rate of urea excretion, since there is no indication that the minus values occur predominantly with low volumes of urine, nor any clear tendency towards a gradual increase over the normal value to successively greater plus values

[3] Addis, T., and Watanabe, C. K., *J. Biol. Chem.*, 1916, xxvii, 267.

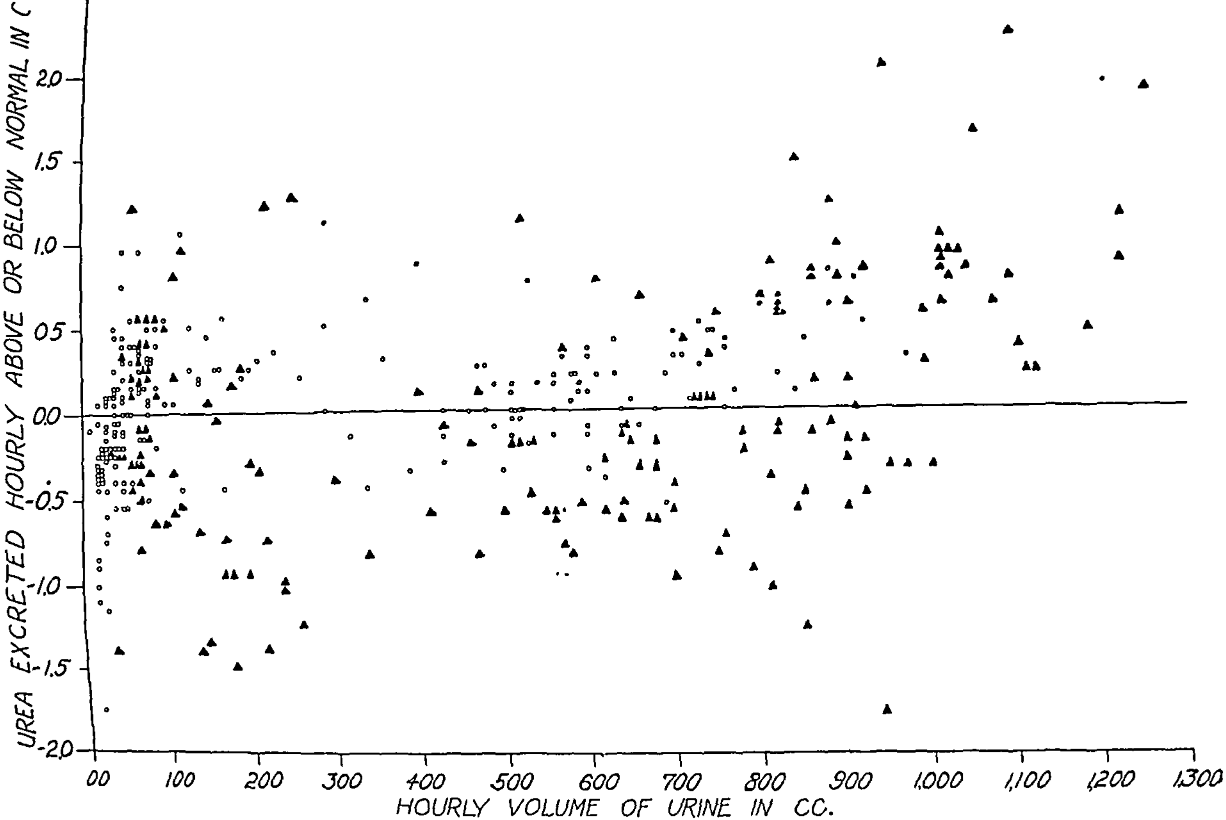

FIG. 1. The absence of any direct relationship between changes in the volume of urine and the rate of urea excretion. Observations on a group of individuals. The circles indicate experiments in which no urea was given, and the triangles those in which urea was administered.

subnormal rates of urea excretion and that when a large quantity of water is drunk the rate of excretion may become greater than normal as the urine volume increases. But these two phenomena appear to be independent of one another. Both may be the result of some common cause, such as an alteration in the blood circulation through the kidneys or a change in the activity of the kidney cells. If changes in the free water content of the blood have an effect on urine volume comparable to that which alterations in its urea concentration have on the rate of urea excretion, it might be possible to demonstrate a parallelism between variations from normal volumes and normal rates under the influence of some such common cause.

CONCLUSIONS.

1. Changes in the volume of urine or in the urea concentration of the urine have no appreciable effect on the rate of urea excretion.
2. When no food or water have been taken for some time, the drinking of large quantities of water is followed by an increase in urine volume and by a synchronous acceleration of the rate of urea excretion which cannot be accounted for on the basis of changes in blood urea concentration. But there is no evidence that this increased rate is a result of the increased volume of urine for the degree of increase above the normal in the rate is quantitatively independent of the degree of increase in volume.

THE PARTITION OF NON-PROTEIN NITROGEN IN THE BLOOD OF FRESH WATER FISH.

By D. WRIGHT WILSON AND EDWARD F. ADOLPH.

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(Received for publication, February 9, 1917.)

The introduction of microchemical methods has made possible many biological investigations which were formerly precluded by the difficulty of obtaining sufficient amounts of material. This has been the case with studies on the blood and urine of fish. The only accurate quantitative chemical data are found in the recent papers of Dr. Denis[1] on marine fish, where she reports results which indicate that the composition of the blood and urine is not only different from that of other animals but may even vary in different species of fish.

It has therefore seemed desirable to extend the studies to fresh water varieties in order to gain further insight into the processes of metabolism in these animals. The present study has included determinations of total non-protein nitrogen, urea, ammonia, amino nitrogen, creatine, and creatinine in the whole blood and in the plasma of several species of fresh water fish. The nitrogen partition and the distribution of materials between plasma and corpuscles displayed several unique relationships. The creatine content of the plasma was greater than that of the corpuscles. Urea was present in surprisingly small quantities in most of the bloods and was in greater concentration in the corpuscles than in the plasma. This unequal distribution is in distinct contrast with the uniform diffusion of the compound throughout mammalian tissues.

We have found that the amino nitrogen constitutes the major portion of the non-protein nitrogen in whole blood and is the largest single component in plasma. The quantities present are

[1] Denis, W., *J. Biol. Chem.*, 1912–13, xiii, 225; 1913–14, xvi, 389.

noted. Whether this indicates fundamental differences in the fish or merely accidental variations due to the condition of nutrition or other factors cannot be decided from the data at hand.

The amounts of urea found in most of our experiments are similar to those observed by Karr and Lewis[2] in the blood of hens. The comparison is mainly of interest by way of contrast for it is well known that uric acid is the chief end-product of protein metabolism in the hen, while fish excrete only very small amounts. The urea in the urine of teleosts constitutes, however, a relatively low percentage of the total nitrogen.

We have found in only a single instance a concentration of urea as great as those reported for marine teleosts by Denis. It is interesting to note that the ganoids (sturgeon and gar) while perhaps more closely related to the marine elasmobranchs than are the teleosts, as indicated by their evolutionary development and cartilaginous skeletons, show no relationship so far as the urea concentration of the blood is concerned.

The variations in the content of urea in the blood from different families of fish exhibit an interesting parallelism with the differences in the osmotic pressures. The blood of the elasmobranchs, which contains relatively enormous quantities of urea (2 gm. per 100 cc.), has an osmotic pressure closely approximating that of sea water[3] ($\Delta = 2.3°$). In the marine teleosts, however, the blood contains but one-hundredth the amount of urea and has a much lower osmotic pressure (average $\Delta = 0.7°$). Our results would indicate that there is a tendency toward a lower content of urea in the blood of several fresh water fish, while the osmotic pressure is, in general, still less ($\Delta = 0.5°$). In connection with the differences in osmotic pressure in these animals it may be observed that the elasmobranchs are dependent upon the surrounding medium for the maintenance of the osmotic pressure of the blood, while the ganoids and teleosts seem to be partially independent of the medium. These facts raise several interesting speculations concerning the mechanisms involved in maintaining the osmotic pressure of the body fluids and the possibility of the diffusion of urea and other substances through the

[2] Karr, W. G., and Lewis, H. B., *J. Am. Chem. Soc.*, 1916, xxxviii, 1615.

[3] Bottazzi, F., *Ergebn. Physiol.*, 1908, vii, 161.

gills. The small amount of nitrogenous materials eliminated by the kidney has already been observed.

The extremely low content of urea in the blood of fresh water fish was further emphasized by the observation that the plasmas contained even less than the whole bloods. The localization of urea in the corpuscles is evident and quite unexpected. Analyses of tissues from various animals by Marshall and Davis,[4] Bang,[5] and Karr and Lewis[2] have shown that urea is uniformly distributed throughout the body, the same in plasma as in corpuscles, and have led to the assumption that urea may readily diffuse throughout the body (wherever sufficient water is present) and be maintained at a strikingly uniform concentration in all tissues. Our results argue against a too general acceptance of such an assumption. Furthermore, the data of Karr and Lewis show that the tissues of the hen may vary considerably in their content of urea. As mentioned above, the amounts found by these investigators in the blood and other tissues of the hen are similar to the quantities found by us in the blood of fish. Hence, it seems possible that there may be a tendency for the cells of the blood as well as other cells of the body to retain urea when its concentration becomes very low.[6]

The percentage of urea·nitrogen to total nitrogen ranges from 4 to 27 in whole blood and from 2 to 29 in plasma. The majority of the values are surprisingly low when compared with other animals and at once lead us to inquire as to what types of nitrogenous compounds constitute the large remaining portion of the non-protein nitrogen.

Amino nitrogen was present in amounts which at once account for most of the non-protein nitrogen of the blood. There were found concentrations of 17 to 34 mg. of amino nitrogen per 100 cc. of whole blood, which constituted from 60 to 80 per cent of the total non-protein nitrogen.[7] These values are considerably higher

[4] Marshall, E. K., Jr., and Davis, D. M., *J. Biol. Chem.*, 1914, xviii, 53.

[5] Bang, I., *Biochem. Z.*, 1915–16, lxxii, 104.

[6] Data have been reported to indicate that under pathological conditions an unequal distribution of urea may be encountered.

[7] A few determinations carried out by the trichloroacetic acid precipitation as described by Bock indicated that the values reported above may be too low. A sample of the whole blood composite from the catfish gave results for both amino nitrogen and total non-protein nitrogen about 50 per cent higher than those tabulated above.

than the figures reported for amino nitrogen in the blood of mammals.[8] Van Slyke and Meyer[9] found 3 to 10 mg. in the blood of dogs, depending on the state of nutrition of the animals. Bock[10] has recently reported intermediate values for bloods of various mammalia.

The plasma contained less amino nitrogen than the whole blood. Calculated from the hematocrit values, the corpuscles were found to contain from three to five times as much as the plasma. György and Zunz[11] and Costantino[12] observed a similar massing of the amino nitrogen in the corpuscles of dogs, although the concentration was much lower in these animals.

The relationship between the amino nitrogen and urea is practically the reverse of that in the blood of higher animals where we find urea the chief nitrogenous constituent. The unusual concentration of amino nitrogen suggests that the amino-acids as such may play a more important part in the intermediary protein metabolism of the fish and possibly constitute an important means for the elimination of nitrogen. The small proportion of total nitrogen accounted for by the materials determined by Denis in the urine of fish (urea, ammonia, uric acid, creatinine, and creatine) lends support to the suggestion.

The relatively high content of ammonia in the bloods examined agrees with the observations of Denis on marine fish. We have not obtained, however, figures approaching the maximum values which she reported. The rapidity with which ammonia develops in blood on standing, together with the fact that several hours are unavoidably required to collect sufficient material for the experiments, would indicate that the minimal values probably represent more exactly the actual amounts of ammonia in the circulating blood.

The content of creatinine in whole blood and plasma was similar to that in mammalian blood. Creatine was present in somewhat larger quantities than has been found in the blood of higher animals by a similar method.[13] The preponderance of creatine

[8] Our figures range even higher than those reported by J. C. Bock, (*J. Biol. Chem.*, 1917, xxix, 191) for the amino nitrogen in the blood of birds.

[9] Van Slyke, D. D., and Meyer, G. M., *J. Biol. Chem.*, 1912, xii, 399.

[10] Bock, *J. Biol. Chem.*, 1916–17, xxviii, 357.

[11] György, P., and Zunz, E., *J. Biol. Chem.*, 1915, xxi, 511.

[12] Costantino, A., *Biochem. Z.*, 1913, lv, 402.

[13] Wilson, D. W., and Plass, E. D., *J. Biol. Chem.*, 1917, xxix, 413.

over creatinine in the plasma of fish corresponds to the relationship of the two compounds in the urine and emphasizes the relative importance of the former in the creatine-creatinine metabolism of these animals at least in so far as concerns excretion.

The variations in concentrations of materials between plasma and corpuscles have been demonstrated in many ways, especially in connection with inorganic constituents and lipoid materials. The importance of such study in connection with the nitrogenous substances has not been sufficiently emphasized. The known compounds making up the non-protein nitrogenous fraction of the blood are for the most part materials of interest chiefly as end-products ready for elimination from the body. The study of the distribution of these substances between corpuscles and plasma as well as between plasma and tissues may assist in solving some of the intricacies of the intermediary metabolism with which the various compounds are concerned.

SUMMARY.

The following constituents were determined in the whole blood and in the plasma of several species of fresh water fish including ganoids and teleosts: total non-protein nitrogen, urea, ammonia, amino nitrogen, creatinine, and creatine.

The urea content of most of the bloods was unusually low. The concentration in the plasma was less than that in the corpuscles.

The amino nitrogen constituted the major part of the total non-protein nitrogen of the blood. The corpuscles contained considerably more than the plasma.

Creatine was present in larger amounts in the plasma than in the corpuscles and was unusually high in the plasma.

CREATINE AND CREATININE IN WHOLE BLOOD AND PLASMA.

By D. WRIGHT WILSON and E. D. PLASS.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, and the Obstetrical Clinic, Johns Hopkins Hospital, Baltimore.)

(Received for publication, February 13, 1917.)

The methods elaborated by Folin[1] for the determination of small quantities of creatine and creatinine have been of great service in quantitative investigations involving these compounds, and the numerous criticisms and modifications suggested as improvements on the original technique are sufficient evidence of their widespread use. But recent discussions[2,3,4] have disclosed several possible sources of error which have been sufficiently emphasized to prevent further confusion.

During the last year we have been interested in the estimation of creatine and creatinine in blood and have encountered several of the difficulties described in recent communications. With these obstacles overcome to our satisfaction, we have determined preformed and total creatinine both in whole blood and in plasma from different animal species, using the original method of Folin and, at the same time, another procedure for total creatinine which we shall describe. Comparisons of the two methods have indicated that the method of Folin may yield inaccurate results for total creatinine, at least when applied to whole blood. The differences observed between the concentrations of creatine in whole blood and plasma from human beings as determined by the newer method emphasize the importance of such differentiation in studies whose object is to correlate the quantities of the

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 475.

[2] McCrudden, F. H., and Sargent, C. S., *J. Biol. Chem.*, 1916, xxvi, 527.

[3] Hunter, A., and Campbell, W. R., *J. Biol. Chem.*, 1916–17, xxviii, 335.

[4] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

substance in blood and urine. A comparison between the concentrations of creatine and creatinine in the plasma and in the urine of the various animals studied has led us to the conclusion that a rough quantitative relationship exists.

The use of Folin's methods for the estimation of creatine and creatinine necessitates the comparison of slight colorations due to small amounts of creatinine in the presence of alkalinized picric acid solutions of relatively high concentration. As considerable errors occur whenever creatinine solutions of different concentration are compared, curves of correction similar to those recently described by Hunter and Campbell[3] were worked out. We have plotted the actual against the theoretical readings, at the same time plotting the logarithms of the concentrations of creatinine along the theoretical axis. The resultant curve fell away gradually from the straight line demanded by theory. Our readings are practically identical with those reported by Hunter and Campbell so that a repetition need not be made. In addition, we have worked out a curve for the 1 mg. standard in 1.2 per cent picric acid and find that our readings do not vary appreciably from the theoretical when reading stronger solutions against the standard, but when reading a solution containing 0.5 mg. of creatinine per 100 cc. against the 1 mg. standard set at 10 mm. an error of 1.5 mm. was observed. The shape of this curve is similar to the others. In working with diluted picric acid solutions, as described in Folin's micro methods for urine, we found no appreciable correction when comparing solutions up to 2 mg. per 100 cc. against the 1 mg. standard, and an error of only 0.5 mm. when comparing 0.5 mg. with the 1 mg. standard.

With the use of the curves described above, the elasticity of the methods is greatly increased in that the necessity for care in choosing standards is in large part obviated. We have demonstrated to our satisfaction many times that the applications of the considerable corrections necessary when reading dilute creatinine solutions in the blood determinations against standards of quite different concentration yield results agreeing with those obtained by comparisons with standards of proper concentration. With the application of these corrections and the personal correction, the colorimetric comparison takes on a greater accuracy than we are justified in assuming exists in the other parts of the methods.

The personal correction in colorimetric readings has recently been emphasized by Folin. It may be mentioned in this connection that our personal corrections were quite different. Under identical conditions, one of us has had a personal correction of 1 mm. and the other of 2 mm. when comparing a 0.2 mg. standard in both cells of the colorimeter. The intensity of the red color and, to some extent, the intensity of the light influence the magnitude of the correction.

Although saturated picric acid solutions are usually assumed to be 1.2 per cent, the solubility of the substance in water may vary from 1 to 1.5 per cent under ordinary changes of temperature in the laboratory. Such variations are sufficient to cause differences of 2 to 3 mm. in colorimetric readings when comparing 0.2 mg. standards. The presence of salts and perhaps of other impurities also influences the solubility. We have therefore thought it advantageous to use solutions of picric acid of known concentration and have made up roughly standardized solutions containing 1.2 per cent picric acid by titration with 0.1 N NaOH, using phenolphthalein as an indicator. Solutions of purified picric acid[5] of this concentration show some tendency to crystallize out at low temperatures while an ordinary preparation of sufficient purity does not. Crystallization may be retarded by introducing sodium chloride into the solutions.

We have observed that heat as well as light causes a decomposition of picric acid with the formation of compounds which yield a red color in alkaline solution. The deterioration of the picric acid solutions, when heated with other substances, is sufficient to affect materially the determination of small quantities of creatine. This being the case, the accuracy of the original method of Folin for the estimation of total creatinine in blood might well be questioned until confirmatory evidence is adduced by the use of another procedure. Hence, we have compared results obtained by the use of the original Folin method and by a procedure based on the removal of protein material by coagulation with heat and acetic acid. The method, described as follows, is merely a combination of several well known procedures: 10 cc. of blood are run into five volumes of boiling 0.01 N acetic acid in a casserole and the solution is boiled for about ½ minute. An equal volume

[5] We have used a method for purifying picric acid similar to the one recently described by Folin and Doisy.[4]

of boiling water is added and the heating continued for about a minute. The solution is filtered through a folded filter and the casserole and paper are washed three times with 15 to 20 cc. of boiling water, rubbing free the material adhering to the dish. 10 cc. of a 15 per cent suspension of aluminium hydroxide are added to the filtrate which is then heated to boiling. Upon filtering again a water clear filtrate is obtained. This is acidified more strongly with 2 to 3 cc. of dilute acetic acid and evaporated on a water bath with a fan, keeping the dry sides of the evaporating dish well above the supporting rings. When concentrated to 2 to 5 cc., the solution is transferred to a 50 cc. Erlenmeyer flask, rinsing the dish several times with small portions of hot water and rubbing with a rubber tipped rod. The final volume of liquid is always approximately 10 cc. 1 cc. of 5 N HCl is added, the flask covered with a watch-glass, and heated on a boiling water bath for 3 to 4 hrs. After cooling, the solution is neutralized with 15 per cent NaOH and 20 cc. of 1.2 per cent picric acid are added. 1.5 cc. of 10 per cent NaOH are introduced, the solution is allowed to stand for 10 minutes, and then diluted to 100 cc. (or 50 cc.) with water. At the same time, 13 cc. of water and 20 cc. of picric acid solution containing a suitable amount of creatinine are introduced into a 100 cc. (or 50 cc.) volumetric flask, 1.5 cc. of 10 per cent NaOH added, and diluted to the mark with water after standing 10 minutes.

TABLE I.

Comparison of Acetic Acid Coagulation with and without Aluminium Hydroxide. Creatinine per 100 Cc.

Source.	Whole blood.		Plasma.	
	Acetic.	Acetic + Al $(OH)_3$	Acetic.	Acetic + Al $(OH)_3$
	mg.	*mg.*	*mg.*	*mg.*
Dog......	2.0	2.0	2.5*	2.1
Cat.......	2.3	2.0	2.7*	1.9
Rabbit....	5.0 5.0 6.6*			
Pig 3.....	5.4* 6.7*	5.2 5.1	6.7*	5.8
" 4.....	4.8* 4.4	4.4 4.4	4.2	3.6
" 5.....	6.1* 6.5* 6.3* 5.9*	5.3 5.3 5.2		
Human...			1.8*	1.5 1.5 1.5

* Some pigment was observed.

When only limited quantities of blood were available, 5 cc. portions were used and the unknown and standard were diluted to 50 cc. It would seem, however, much more desirable to use quantities of blood which yield at least 0.2 mg. of creatinine. Curves similar to those mentioned earlier in this paper have also been worked out and used in these determinations.

The use of aluminium hydroxide is a distinct advantage though not absolutely necessary. The small amounts of protein which escape coagulation are removed by this means and the solution remains colorless even after heating with hydrochloric acid to convert creatine into creatinine. When a perfectly water clear filtrate results from the coagulation with acetic acid, little or no pigment may be formed during the conversion of creatine into creatinine, but usually a sufficient quantity is produced to interfere seriously with the determination. In Table I may be found the results of determinations made with and without the use of aluminium hydroxide on specimens of the same bloods. It will be noticed that whenever pigment formation was observed after the use of acetic acid alone, the results are higher than when aluminium hydroxide was also used. When no pigment was formed the results are the same. These observations, together with control determinations with creatine and creatinine solutions, show that the aluminium hydroxide does not remove appreciable quantities of either substance or interfere with the colorimetric estimation. The ease with which duplicates may be obtained with the use of aluminium hydroxide and the difficulties encountered when it is not used emphasize its value.

The method described above was used in connection with a comparative study on the creatine and creatinine content of whole blood and plasma from different animals. The preformed and total creatinine were also determined by the original procedure of Folin with and without preliminary hemolysis with water. The data which we have collected may be found in Table II. Most of the determinations on the blood from lower animals and a few of the human bloods were done in duplicate.

The results of the analyses of total creatinine in whole blood by the two different procedures show great divergence. In every case the picric acid method yielded values higher, at times

TABLE II.

Total and Preformed Creatinine in Whole Blood and Plasma by the Picric Acid and Acetic Acid Methods, in Mg. per 100 Cc.

Source.	Whole blood.					Plasma.				Remarks.
	Creatinine		Total creatinine.			Creatinine.	Total creatinine.			
	Not hemolyzed.	Hemolyzed.	Not hemolyzed.	Hemolyzed.	Acetic.	Picric.	Picric.	Acetic.	Creatine.	
Man (P.).....	2.1	2.4	7.2	7.8		1.4	1.9			Normal.
" (P.).....						1.3	2.0			"
" (M.).....	2.1	2.9	9.2	11.0		1.4	2.2			"
" (W.).....		2.3		5.5	2.9	1.4	1.8	1.4	0	Creatine-free diet. Normal.
Woman (H.)..		3.1		6.5	3.0	1.2	1.8	1.2	0	Normal.
"		2.6		5.7	3.0	1.1	1.9	1.2	0.1	Mixed specimen. Normal.
" (T.)..		1.2		6.8		1.1	1.6	1.6	0.5	In labor
" (B.)..						1.3	1.9	1.7	0.4	" "
" (S.)...	1.8	2.5	4.9	5.5	3.8	1.1	1.6	1.3	0.2	Eclamptic.
Infant (M.)...						1.1	1.9	2.0	0.9	New-born. (Serum.)
" (S.)....						1.1	1.9	2.0	0.9	" "
" (B.)...						1.0	1.9	2.0	1.0	" "
Dog 2........	1.7	2.3	5.0	4.3	2.0	1.7	2.4	2.1	0.4	
" 3........						1.0	1.5	1.3	0.3	
Cat 1.........	2.7	2.8	6.7	7.1		2.4	3.8	4.0*	1.4	Urine: creatinine 153 mg., creatine 21 mg. per 100 cc.
" 2.........	1.7	1.8	4.7	4.3	2.0	1.3	1.9	1.9	0.6	Fasted 24 hrs. Urine: creatinine 191 mg.; creatine 24 mg. per 100 cc.
Rabbit 1......	2.4	3.8	7.5	7.7	5.0*	1.7	5.4		3.7	
" 2......		3.1		10.5	8.1	2.0	8.5	8.3	6.3	Tartrate nephritis. Urine: 4 cc. contained 1.6 mg. of creatinine and 6.2 mg. of creatine.
Pig 2.........	2.0	2.1		6.5	4.7	1.9	5.3	5.2	3.3	
" 3.........		2.0			5.2			5.8	3.8	
" 4.........	1.8	2.0	6.0	5.8	4.4	1.8	4.0	3.6	1.8	
Hen 1.........	0.9	1.1	7.1	7.1	2.4*	0.8	2.3	2.6*	1.8	
" 2.........	1.2	1.2	5.0	5.1	2.4	1.2	2.4	2.5	1.3	

* No $Al(OH)_3$ used. Solutions colorless or nearly so.

100 per cent higher, than those of the acetic acid procedure. When applied to plasma the two methods were in closer agreement. Hence it would appear that something is present in the corpuscles which remains in the picric acid solution, so that after autoclaving an atypical color development is observed when the solution is made alkaline. We have often observed that the color development is much more rapid in these solutions than in the standard creatinine solutions and the development continues after the standard solution has nearly reached its maximum color (*i.e.*, after 10 minutes). Moreover, the color of the blood solution is different from that of the standard solution, possessing a definite brownish tinge and making the color comparison more difficult.

The application of the method of Folin to unhemolyzed plasma yields more satisfactory results. The color development is much more typical of pure creatinine solutions, and the agreement of the results with those of the acetic acid procedure, where the color development appears to be typical, lends support to the accuracy of the method. We believe that the quantities of total creatinine obtained by the use of the acetic acid procedure on blood should be considered maximum and not minimum values. It must therefore be concluded that the method of Folin is unsatisfactory for use with whole blood.

The rapid and continuous development of a color of different quality is also often noticed in the determinations of preformed creatinine, especially in whole blood. In one experiment, where actual comparisons were made, the creatinine concentration of whole blood, calculated from readings taken at the end of 10 minutes was 1.7 mg. per 100 cc.; 1 hour later, when read against the same standard, the calculated creatinine concentration was 2.5 mg., an increase of 50 per cent. The preformed creatinine values are usually higher when the blood is hemolyzed before the addition of picric acid. Whether this increase may be due to a liberation of creatinine which is otherwise occluded in the voluminous precipitate or merely the liberation of color-producing material not creatinine cannot be determined from the data at hand, but the latter assumption seems to us the more plausible.

Our observations on the preformed creatinine in the whole blood of different animals range from 1 to 3 mg. per 100 cc.,

quantities similar to most of those reported by other investigators. The blood of hens seemed to contain practically as much creatinine as that of other animals. This is contrary to the observations of Folin and Denis,[6] but more in accord with the data reported by Myers and Fine.[7]

The determinations of preformed creatinine in the whole blood are frequently higher than those in the plasma but the variations are sufficiently small to be ascribed to the anomalous color development when whole bloo' is used. It would seem probable that creatinine is equally ..tributed between plasma and corpuscles.

By the use of the acetic acid method described earlier in the paper, we have found from 2 to 5.2 mg. of total creatinine per 100 cc. in the whole blood of various animals which were presumably normal. Human blood contains about 3 mg. per 100 cc. These quantities are much lower than practically all of the reported data, for the reason that the picric acid method has been almost universally used. Shaffer[8] reports a single determination on the whole blood of a dog by a method of coagulation with heat and acetic acid. As he obtained 7.4 mg. per 100 cc., we should conclude that probably pigments interfered with the accuracy of the determination. Beker[9] analyzed the whole blood from three species of animals by coagulating with heat and acetic acid, precipitating with lead acetate, concentrating, and autoclaving with HCl. His results are much more in accord with ours. He found from 1.9 to 2.4 mg. per 100 cc. of dog blood, 2 mg. in pig blood, and from 1.9 to 2.7 mg. in ox blood.

In most instances, the plasma contained practically the same quantity of total creatinine as the whole blood. The human plasma, however, contains less. It appears that the corpuscles in human blood contain appreciably more total creatinine than does the plasma. Owing to the lack of entirely satisfactory data on preformed creatinine especially in whole blood it is impossible to say definitely whether the material stored in the corpuscles is creatine or creatinine. It would seem most prob-

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1914, xvii, 487.

[7] Myers, V. C., and Fine, M. S., *J. Biol. Chem.*, 1915, xxi, 583.

[8] Shaffer, P. A., *J. Biol. Chem.*, 1914, xviii, 525.

[9] Beker, J. C., *Z. physiol. Chem.*, 1913, lxxxvii, 21.

able, however, that creatinine is present in practically equal amounts in corpuscles and plasma but the corpuscles may store some creatine under normal conditions.

The determinations of preformed creatinine in plasma seem to be sufficiently accurate to warrant a consideration of the quantities of creatine calculated by subtracting the values of the preformed from the total creatinine as determined by the acetic acid method. These figures are also tabulated.

A comparison of the relationship between creatine and creatinine in plasma and in urine seems to offer more convincing results than any such comparison of values obtained by the older methods on whole blood. We have found little or no creatine in adult human plasma. This observation offers an explanation for the fact that creatine is seldom found in the urine of normal men and in small and variable amounts in the urine of normal women. Whether the method is sufficiently accurate to show the presence of creatine in the plasma when, and only when, small quantities are eliminated is uncertain, but our few determinations point to that possibility. Infants, who are known to eliminate relatively large quantities of creatine, have in their plasma practically as much creatine as creatinine.

The quantities of creatine in the plasma of the dogs which we have examined are barely appreciable. The urine from these animals was not examined, but it is well known that creatine is excreted irregularly by normal dogs. More creatine was found in the plasma of cats, one of which had fasted 24 hours. A small amount of creatine was present in the urine of these animals.

A still larger quantity of creatine was found in the plasma of a rabbit. The animal was presumably normal but its nutritive condition was not determined. The recent work of Underhill[10] shows the sensitiveness of these animals to conditions causing the excretion of creatine. The second rabbit had received sodium tartrate several days previously but was not anuric. Daily examinations of the urine showed only a slight diminution in the output of creatinine, while the concentration in the blood was practically unchanged. There seemed to be, however, a definite accumulation of creatine resulting in a correspondingly greater

[10] Underhill, F. P., *J. Biol. Chem.*, 1916, xxvii, 127.

excess of creatine over creatinine in the plasma. A similar relationship was observed in the urine in which the creatine constituted over three-fourths of the total creatinine.

The different specimens of plasma from pigs contained different concentrations of creatine. We observed variations from 1.8 to 3.8 mg. per 100 cc. of plasma, quantities equal to or greater than the preformed creatinine. Here, again, the possible relationship of the quantities of creatine in the blood and urine is of considerable interest. McCollum and Steenbock[11] have shown that the pig normally excretes creatine and the amount eliminated is dependent to a considerable extent upon the protein ingestion. They have observed that creatine may constitute as much as 40 per cent of the total creatinine eliminated. The large and variable quantities of creatine eliminated by the pig may be dependent upon similar variations in its concentration in the plasma.

Creatine is present in larger quantities than creatinine in the plasma of hens, but the excess is not so great as might be expected from the analyses which have been reported on the urine of birds. Paton[12] states that "creatine takes the place of creatinine in the urine of the bird," but leads the reader to conclude that he may have detected small amounts of creatinine which were assumed to be formed by his analytical procedure. Thompson[13] has recently reported analyses of the urine from ducks, which would indicate that the creatinine elimination is not negligible but is from one-third to one-fifth as much as the creatine. While these observations support Paton's general conclusion, the elimination of creatinine as well as creatine in the urine is more in accord with our point of view that the presence of appreciable quantities of creatine or creatinine in the plasma leads to their excretion.

The results which we have obtained, first, by the examination of unhemolyzed plasma and, second, by the substitution of a more exact method for estimating total creatinine permit a much more exact and rational viewpoint of the quantitative relationships

[11] McCollum, E. V., and Steenbock, H., *J. Biol. Chem.*, 1912-13, xiii, 209.

[12] Paton, D. N., *J. Physiol.*, 1910, xxxix, 485.

[13] Thompson, W. H., *J. Physiol.*, 1916, l, p. xxii.

between creatine and creatinine in the blood and urine. The older observations led to the anomalous conclusion that creatine is present in considerable quantities in the blood of man while little if any normally escapes into the urine, but that under certain slightly abnormal conditions it may be eliminated in considerable quantities. Moreover, the variations in the concentration of creatine in the bloods of different animals showed no relationship to the relative amounts of creatine excreted. Our results would indicate that when appreciable quantities of creatine (or creatinine) are present in the plasma, some is eliminated in the urine and the amount eliminated bears a rough quantitative relationship to the concentration in the plasma. Such an hypothesis seems to be capable of satisfactory proof with the use of the procedures which we have employed in this investigation. Our observations also suggest that, as the concentrations of creatine in plasma and corpuscles of man are different, they should be studied independently.

SUMMARY.

The method of Folin for the determination of total creatinine is unsatisfactory for use with whole blood. A different procedure is suggested.

Data are presented for preformed and total creatinine in whole blood and plasma from different species of animals.

Adult human plasma contains only traces of creatine. Appreciable quantities are present in the plasma of infants. The plasmas of the pig and hen contain larger quantities.

A characteristic relationship appears to exist between the concentration of creatine in the plasma and its elimination in the urine.

STUDIES IN BENCE-JONES PROTEINURIA. II.

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Elimination of the Protein.

A striking feature of Bence-Jones proteinuria is the free passage of large quantities of the protein through a kidney which holds back the normal serum proteins.

Most previous investigators have found the kidney of animals impervious to the Bence-Jones protein, although Boggs and Guthrie[1] found that sometimes rabbits excreted it after intravenous injection. We injected subcutaneously 40 cc. of Bence-Jones urine into each of two dogs, weighing respectively 8 and 9.5 kilos. All urine passed during the following 3 days was entirely free from protein of any kind (ferrocyanide and acetic acid, heat, HCl). On the 3rd day one of the dogs was etherized and into the femoral vein were injected 30 cc. of Bence-Jones urine. The urine of this dog for the next 2 days showed no protein of any kind. The amounts of Bence-Jones urine taken for injection represented in each instance a little more than 1 gm. of the protein.

To try the effect of larger quantities we etherized a 14 kilo dog and injected into a vein of the leg 5 gm. of Bence-Jones protein dissolved in 200 cc. of slightly alkaline Ringer's solution. The urine of this dog before injection showed just a trace of albumin; but as this is not uncommon in dogs and as the animal was strong, active, and apparently perfectly well, we did not reject him on that account. When coming out of the ether the animal urinated, and again about 20 minutes later. Both of these urines were entirely similar to that obtained before injec-

[1] Boggs, T. R., and Guthrie, C. G., *Am. J. Med. Sc.*, 1912, cxliv, 803.

tion, and free from Bence-Jones protein. The urine found the next (2nd) day produces a heavy white cloud when heated with a drop of acetic acid; this cloud almost disappears upon boiling and reappears upon cooling, and this can be repeated. The characteristic acid reactions are also given in strong typical fashion.[2] Evidently we here have a marked excretion of Bence-Jones protein. Upon estimation it was found that the urine contained 0.16 per cent, or for the total amount of urine 1.47 gm. of Bence-Jones protein. When the urine was carefully coagulated at boiling, allowed to cool, and filtered, the clear filtrate (which could no longer at any reaction or temperature be made to give cloudiness on heating) gave some opalescence when saturated with ammonium sulfate; and also gave moderate reactions with ferrocyanide and Roberts' reagent. While these positive tests in the filtrate may have been caused by failure to remove all of the coagulable protein, it seems more probable that there was a small amount of proteose present. Whatever the nature of this filtrate protein, its total amount was evidently very small in comparison with the coagulable protein. The urine found on the 3rd day was nearly free from protein. Spiegler and ferrocyanide tests both indicated traces, but these reactions were little if any stronger than this dog normally showed; there was no reaction for Bence-Jones protein. The urine found on the 4th day showed only traces of albumin, no stronger than before the experiment, so the dog was removed from observation. It is evident that rather less than one-third of the injected Bence-Jones protein was eliminated during the first 24 hours, and that after that time practically no more was recovered. Even granting that a very small amount of proteose was present in the urine of the first period, not more at most than one-third of the protein is accounted for; what became of the other two-thirds we are unable to say; but as even when injected directly into the blood stream it was neither eliminated as Bence-Jones protein nor digested and eliminated as proteose, it does not seem unreasonable to assume that it is promptly fixed by the blood cells or combined with something in the plasma, and subsequently made use of or catabolized in the organism.

When the dog has previously been moderately poisoned by uranium nitrate, the injection of considerable quantities of the

[2] Taylor, A. E., and Miller, C. W., *J. Biol. Chem.*, 1916, xxv, 281.

protein produces very different results. A healthy dog weighing 6.2 kilos was injected subcutaneously with 0.03 gm. of uranium nitrate, 4.8 mg. per kilo. By the next (2nd) day the urine showed a moderate amount of albumin, and on the 3rd day the dog received (under ether anesthesia) into a vein of the leg 8 gm. of Bence-Jones protein dissolved in quite alkaline Ringer's solution. After coming out of the ether he vomited twice. The filtrate of this vomitus could not be made to coagulate by heat and seemed free from Bence-Jones protein. The urine found on the 4th day contained some albumin by boiling, but hardly as much as before the protein was injected. There was no trace of coagulation when heated for an hour at 58° with a drop of weak acetic acid, and the HCl test was negative; evidently no Bence-Jones protein was present. There was, however, a very heavy reaction to ferrocyanide and acetic acid totally out of proportion to the amount of protein coagulated on boiling. The urine found on the 5th day had the same characteristics, and as the dog seemed to be moribund he was killed with chloroform, and about 30 cc. of urine were taken from the bladder. This gave the same reaction as before. Macroscopic examination of the stomach, liver, and gall-bladder revealed nothing, while the kidneys showed moderate inflammation.

This experiment was repeated substituting Bence-Jones urine for the separated protein. A dog weighing 12 kilos was injected subcutaneously with 0.075 gm. of uranium nitrate, 6.2 mg. per kilo. The next (2nd) day the urine contained a moderate amount of albumin by the heat, Spiegler, Roberts', and ferrocyanide tests. The urine found on the 3rd day reacted similarly and under ether anesthesia the dog received into a vein of the leg 250 cc. of Bence-Jones urine, representing nearly 7 gm. of protein. This dog also vomited after the ether, and the vomitus was, like that of the previous animal, free from Bence-Jones protein. The urine of the 4th day contained less albumin than before and no Bence-Jones protein. The reaction to ferrocyanide is very strong, and there is also a strong biuret test. To one part of the urine were added ten parts of absolute alcohol; this was allowed to stand an hour and filtered. To a portion of the filtrate was added water, evaporated down to small volume on the water bath, when it gave a strong biuret reaction. The

urine of the 5th day gave the same result. Also a portion of this urine was saturated with ammonium sulfate, and a drop of acetic acid added; the result was a heavy precipitate, and the clear filtrate gave a strong biuret reaction. Another portion of the urine was saturated with ammonium sulfate and allowed to stand with excess of the sulfate for 36 hours, when as before the clear filtrate gave a strong biuret reaction. The precipitate produced by the ammonium sulfate was in each instance many times heavier than the heat coagulum. The 6th day the dog died.

From these two experiments it would seem that the uranium dogs have lost their normal power of utilizing the Bence-Jones protein, and that the latter is so energetically hydrolyzed that a portion even escapes precipitation by ammonium sulfate.

It might be objected that the uranium itself or the etherization were in some way responsible for the appearance of digestion products in the urine. In order to test this point we injected another dog with uranium nitrate as before, but gave no protein. The urine for 2 days following showed no proteose. On the 3rd day the dog was etherized for 20 minutes, and the urine for the following 3 days still showed no proteose. This dog was the 14 kilo animal previously used in the experiment of injecting 5 gm. of protein. He was, however, entirely well as far as could be observed, and in the best of spirits. The amount of uranium nitrate used was 0.09 gm., 6.4 mg. per kilo; and it is interesting to note that although more uranium was used than in the other experiments this dog appeared to have practically recovered by the 6th day, the albumin having almost disappeared from the urine, and he was in the best of spirits with excellent appetite. He remained under our observation for a couple of weeks and was apparently absolutely well.

Another dog weighing 9.7 kilos was injected with uranium nitrate, using 0.06 gm., 6.1 mg. per kilo. On the 2nd day there was considerable albumin, and on the 3rd day also. On this day under ether anesthesia we injected into a vein 200 cc. of normal urine. The urine of the 4th, 5th, and 6th days showed no proteose. This animal recovered rather slowly, and it was 10 days before he again seemed in normal condition.

DISCUSSION.

These experiments indicate that the normal dog is able to utilize or catabolize a moderate amount of Bence-Jones protein, even when this is injected rapidly into the circulation; but that a limit is soon reached beyond which the excess protein is promptly excreted in unchanged condition. In dogs suffering from moderate uranium poisoning this power of utilization appears to be lost; though on the other hand no Bence-Jones protein is excreted as such, but only after being broken down to proteose. It is interesting that the dog receiving the largest dose of uranium (6.4 mg. per kilo) but no urine or Bence-Jones protein promptly recovered; that the dog receiving uranium nitrate and then normal urine showed more serious toxic symptoms but did recover; and finally, that of the two dogs receiving uranium nitrate and then Bence-Jones protein and freely excreting proteose, one died on the 6th day, and the other was moribund when he was killed on the 5th day. Since the Bence-Jones protein is not toxic to the healthy dog,[2] and since, apart from our own results, this amount of uranium would not be expected to cause rapid deaths,[3] it would seem that the uranium causes some change in the metabolic power such that the normally harmless or perhaps useful protein rapidly becomes hydrolyzed with unfavorable results. When dealing with uranium poisoning, one's attention is naturally directed toward the kidney; but in these experiments the peculiar effect of the uranium on the elimination of the protein might depend rather on a general toxic influence; possibly some powerful ferment may have appeared in association with the uranium intoxication, with the result of protecting the organism from the breakdown products of the poisoning; and this ferment, while successful in saving the animal from the uranium, is so active that as soon as an abnormal protein is introduced it causes him to be overwhelmed by proteoses formed from the injected protein faster than they can be eliminated. This is the

[3] Thus W. de B. MacNider (*J. Pharm. and Exp. Ther.*, 1913, iv, 491) in his studies concerning elimination by the uranium kidney after anesthetics and diuretics found that it required 6.7 mg. per kilo injected twice to cause the "desired changes in the kidney without the undesirable gastro-intestinal complications."

general type of explanation one would formulate on the basis of Vaughan's hypothesis.

It will be observed that the healthy dog which eliminated a portion of the injected Bence-Jones protein received only 0.36 gm. of the protein per kilo, whereas the uranium dogs received respectively 1.29 and 0.58 gm. per kilo; so there is here at least no evidence in favor of the supposition that a kidney permeable *to normal serum proteins is on that account more easily passed* by the Bence-Jones protein. On the other hand, the conditions of uranium poisoning are not sufficiently similar to those of a chronic nephritis to make positive statements, and we have not succeeded in finding a dog with a natural nephritis upon which this question could be more exactly studied.

The ease with which Bence-Jones protein passes through a kidney impermeable to the normal proteins is almost certainly not due to the molecule being small in size, since nearly all the more recent studies indicate that we have to deal with a higher protein of large molecular weight. For example, Hopkins and Savory, after careful amino-acid determinations state as their opinion[4] that: "Bence-Jones protein yields all those amino-acids which are to be obtained from typical proteins, and is therefore not a fractional product arising from the partial breakdown of protein in metabolism;" and this view is entirely in accord with the conclusions previously arrived at[2] on other grounds by two of us. What may be the nature of the physicochemical adjustment permitting this selective action on the part of the kidney we venture no surmise.

Origin of the Protein.

Two sources suggest themselves: The protein may be a special product of the tumor cells of the myeloma; or it may be a normal or possibly aberrant stage in the synthesis of some body protein, the completion of which is interfered with by deficiency of some necessary condition.

The first of these assumptions is doubtless the more natural, although there seems little direct evidence to support it. If the protein is in fact the product of the tumor cells we should expect to

[4] Hopkins, F. G., and Savory, H., *J. Physiol.*, 1911, xlii, 189.

find at least small amounts of it in the diseased marrow. Reach[5] examined the bones with negative results, but found the protein in the spleen, which organ contained a neoplasm similar to those found in the bones. However, Hopkins and Savory[4] found no trace of the protein in the bone marrow; and when the marrow was injected into rats none of the protein was found in the urine of these animals. When our case came to autopsy we were permitted to take only small specimens of the bones for microscopic examination, and were therefore not able to test this question ourselves. Rosenbloom[6] has suggested an osseo-albuminoid origin for the protein. The strongest argument in favor of the protein being a special product is the unusual amino-acid composition, the phenylalanine and tyrosine together amounting to over 9 per cent of the whole, which, as Hopkins and Savory point out, is "considerably higher than any yet described for a blood or tissue protein. Judging, indeed, by our figures as a whole, the protein would seem to stand by itself." On the other hand, the surprisingly large amounts excreted would rather indicate some more general source. As was previously pointed out[2] the biologic reactions of this protein relate it closely to the normal serum proteins, a conclusion further substantiated by Massini's complement fixation experiments.[7] Our ignorance of tumor composition and of their products, if any, or of the exact degree of foreignness experimentally limiting biologic reactions, makes it impossible to be dogmatic; but the conclusion may well be reserved whether a protein behaving biologically as does this is really a special, and to the organism previously unknown, substance.

If we were to assume that in the diseased marrow something was lacking which prevented the complete synthesis of a normal body protein, then by supplying to Bence-Jones protein normal bone marrow and excess of amino-acids we might, theoretically at least, observe the building up of a protein of higher coagulation point. We at first proposed to carry out such an experiment by perfusing normal dog bone with a solution of Bence-Jones protein and amino-acid solution. After a large amount of

[5] Reach, F., *Deutsch. Arch. klin. Med.*, 1905, lxxxii, 390.
[6] Rosenbloom, J., *Arch. Int. Med.*, 1912, ix, 236.
[7] Massini, R., *Deutsch. Arch. klin. Med.*, 1911, civ, 29.

preliminary experimentation we were forced to abandon this, as we were unable to devise and carry out any scheme that gave promise of reliable quantitative results; and since in this experiment we are sure to have both Bence-Jones and normal protein, it is necessary to operate quantitatively in order to learn whether the latter protein is being increased at the expense of the former. We then tried the following experiment *in vitro*. An amino-acid solution obtained by tryptic digestion of dog muscle was heated to boiling, filtered, and cooled, and to 200 cc. of it were added 2.25 gm. of Bence-Jones protein as well as the ground up bone marrow from the long bones of a large dog, removed as aseptically as possible and used perfectly fresh. A portion of this mixture was put into the incubator with toluene, and the other portion used to estimate under definite previously determined salt and acid relations the respective content in Bence-Jones and high coagulation point protein. The portion in the incubator was allowed to remain 7 days, then removed and analyzed as before. The results showed that only a trace of the Bence-Jones protein remained, but that nearly all of the serum protein had also disappeared. Therefore any possible synthetic action was entirely lost in the active digestion which had broken down nearly all of the coagulable protein. When we remember the comparative ease with which various ferments accomplish digestion and the well known difficulties surrounding the production artificially of biologic synthesis, this result will cause no surprise; and we feel that this experiment can only be regarded as leaving the question unsettled.

Course of the Case Previously Reported.

The improvement in the condition of the patient, previously reported,[2] up to March 1, 1916, proved to be only temporary. After this he failed at first slowly, then more rapidly, all the while maintaining the excretion of Bence-Jones protein at and finally above the previous high level. In addition there were soon added at first small then larger quantities of serum albumin. As we were unable to devise a process for separating with any precision the two kinds of protein without at the same time causing material denaturation, this change in the character of the excretion unfortunately compelled us to abandon a series of ex-

periments which had been planned along biologic lines to obtain more exact information as to which of the proteins of the normal organism the Bence-Jones body is most closely related to. The patient died in October, 1916, and for several months before that time no work was done by us except an occasional examination of the urine.

Autopsy.—The only noticeable abnormality of the viscera was several old healed lesions of the lungs. The pleural cavities contained considerable clear straw-colored fluid. The long bones were not affected; but in the bones of the pelvis, ribs, and vertebræ the marrow occupied a much greater space than usual with marked thinning of the walls; the body of the sacrum was so spongy as scarcely to resemble bone at all, and the bony structure of the ribs in some places was not over 0.5 mm. in thickness. The subsequent histologic examination showed undoubted myeloma, a fact of interest to clinicians since the x-ray had indicated no osseous abnormality.

Besides the specimens for histologic examination, which will elsewhere be reported, we obtained urine from the bladder, blood from the heart, and fluid from the pleural cavities. The *urine* presents the same depth of color and other appearance as usual, but contained 3.6 per cent of Bence-Jones protein, which was more than we had ever before found; and there was also considerable albumin. The *pleural fluid* gave well marked reactions for Bence-Jones protein, and upon estimation was found to contain 0.11 per cent. The *blood* after rubbing down the clot with water and pressing through the filter gave a well marked test with HNO_3 and heat,[2] also the filtrate from boiling coagulation clouded strongly upon cooling. The amount of blood available was not sufficient to make satisfactory determinations, but the protein appeared to amount to about 0.2 per cent, which is confirmed by the fact that the qualitative tests were definitely stronger than in the case of the pleuritic fluid similarly diluted. Because hemoglobin is coagulated or decomposed at about the same temperature at which Bence-Jones protein coagulates, the latter cannot be estimated in the presence of the former by the usual method; it is necessary to bring the whole to boiling and then filter through a hot filter and weigh the protein which separates on cooling. This process requires a large amount of material, gives

results which do not agree well, and apparently the figures are always too low.

From these autopsy findings it is clear that the protein circulates freely throughout the body.

SUMMARY OF BOTH PAPERS.

1. Brief clinical notes of the case are given.

2. A number of reactions of the Bence-Jones protein found in this case, together with methods of identification, are described.

3. A method of separating the protein and of preparing a protein-free urine is also described.

4. Anaphylactic sensitization is accomplished best by the separated protein, less well by the same amount of protein as found in the native urine. Some factor exists in the native urine which prevents the protein contained in it from exerting its normal sensitizing action; and this factor is thermostabile at 55°, but is destroyed by heating to near the boiling point or by such chemical manipulation as described in our method for preparing protein-free urine. Subsequent anaphylactic intoxication is shown equally by the native urine and by the separated protein.

5. The protein shows no direct toxicity.

6. The separated protein is digested with great ease by both pepsin and trypsin.

7. The protein is no proteose, but a higher protein of definite biologic stamp.

8. It is of endogenous origin, and might be derived either from the tumor cells of the myeloma, or produced through an interrupted or aberrant synthesis of some normal body protein. The biologic indications of close relationship to the normal blood proteins, and the enormous quantities produced would seem to favor the second alternative. The question remains obscure.

9. When we allowed Bence-Jones protein, normal bone marrow, and an amino-acid solution to remain together for 7 days nearly all coagulable protein had disappeared. In the presence of this active digestion we were unable to learn whether any synthesis had occurred or not.

10. Normal dogs can utilize or catabolize moderate quantities of Bence-Jones protein, but a limit is soon reached beyond which the protein is promptly excreted in unchanged condition.

11. In dogs suffering from moderate uranium poisoning this power of utilization is lost, and the Bence-Jones protein is energetically hydrolyzed and eliminated as proteose.

12. Moderate doses of uranium nitrate which provoke only moderate symptoms rapidly become fatal when Bence-Jones protein is injected into the circulation. An explanation of this in harmony with the Vaughan hypothesis would be that some ferment arises in association with the uranium intoxication protecting the organism from breakdown products of the poisoning, and that the ferment is so active that as soon as an abnormal protein is introduced the animal is overwhelmed by the proteose formed from the injected protein.

13. At autopsy the bones of the pelvis, ribs, and vertebræ were found involved in extensive myeloma notwithstanding that the clinical findings during life were negative.

14. Urine taken from the bladder at autopsy showed 3.6 per cent of Bence-Jones protein, besides considerable albumin; the pleural fluid showed 0.11 per cent of Bence-Jones protein; and the blood appeared to contain about 0.2 per cent of Bence-Jones protein. Evidently the protein circulates with great freedom throughout the body.

THE DETERMINATION OF CHOLESTEROL IN BLOOD.

By W. R. BLOOR.

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(Received for publication, February 27, 1917.)

Weston[1] has recently reported results obtained by the use of my method for the determination of cholesterol in blood. These results are inconsistent and justify his finding that in his hands the method was useless. On the other hand, the method has given consistent results in this laboratory in various hands for over a year and is known to be in use in other laboratories where a thorough testing out before adoption would be a matter of course. In published work on the subject Mueller[2] has obtained results similar to mine in a comparison of the method with that of Autenrieth and Funk and has reported the alcohol-ether method of extraction "most excellent." Denis[3] has reported essentially the same values for normal blood as I had obtained. While it could hardly be expected that any method would find acceptance at all hands, this method seemed to have the advantages of simplicity in principle and ease and rapidity in operation, with the further advantage that the same alcohol-ether extract may be used for the determination of other blood lipoids. It was desirable therefore to find out if possible the reason for Weston's inconsistent results, both for the justification of those already using the method and to correct if necessary the directions so as to prevent further trouble with it.

Experience has taught that in cases of this kind the most frequent cause of failure is a use of the method in some other way than that directed or intended. It is a fact well known to chemists that few methods are flexible enough to be used in any

[1] Weston, P. G., *J. Biol. Chem.*, 1916–17, xxviii, 383.

[2] Mueller, J. H., *J. Biol. Chem.*, 1916, xxv, 549.

[3] Denis, W., *J. Biol. Chem.*, 1917, xxix, 93.

other way than that worked out by the originator. While Weston's description of his procedure is brief and lacking in essential details, there is sufficient to show that he has probably violated both the principle and the expressed directions. Thus in his version of the method, after describing the extraction with alcohol-ether, bringing to 100 cc., and filtering, the filtrate was divided into two parts, the first part was evaporated to dryness, and the residue "taken up with chloroform." The second was treated with potassium hydrate and then as in the Weston-Kent method. Two points may be noted here; first, that the filtrate is not the whole extract—it is an indefinite volume depending on the amount held back by the precipitate and filter paper, and generally about 90 cc.—so that what is obtained by taking half of it is about 45 per cent of the whole extract; second, that while complete extraction of the residue from half the extract is possible, and is desirable in certain cases, it is a much more difficult and time-consuming procedure than complete extraction of the residue from 10 cc. It does not follow at all that since the cholesterol from a small residue may be extracted by a few minutes' boiling with chloroform the larger residue can be extracted by a similar short treatment. It is evident that failure to get a good extraction is probably an important cause of the inconsistent results, from the fact that lower values are obtained from Solution B which contains 50 per cent more cholesterol than Solution A. Whether the extraction of the serum with alcohol-ether was also at fault is not apparent, but it is evident that it was not always so, for the values which he obtained from the alcohol-ether extract saponified and determined in his own way are consistent and in fair agreement (when increased by 10 per cent to cover the error in aliquot) with those obtained by the various other methods. Thus for Solution A the reported value is 173 mg., and for Solution B 270 mg. These values increased 10 per cent would be 190 and 297 mg., which compare well with the values of 185 and 285 mg. obtained by the other methods. It is perhaps significant that these values obtained by his own method, with which he would presumably be most familiar, are the only consistent results obtained by the use of any part of my method.

My directions for the second part of the procedure, the pro-

duction of the color, call for the cholesterol from 10 cc. of alcohol-ether extract—ordinarily about 0.5 mg. (volumes to contain this amount are specified in later use of the method[4])—dissolved in 5 cc. of chloroform and compared with 0.5 mg. of cholesterol also in 5 cc. of chloroform; that is, the standard and test solutions are of about the same strength. They are also treated with the same amounts of reagents and are otherwise under parallel conditions—a plan of procedure which has been followed in all the methods for blood lipoids and which is believed to be the safe one for work in a new field. In Weston's version of my procedure a corresponding amount of serum extract is used for the test solution, but for the standard he used a solution containing 2.7 mg. of cholesterol in 10 cc. of chloroform—a standard approximately two and one-half times as strong as the test. For this strong solution the same concentration of acetic anhydride and sulfuric acid was employed as for the much weaker test solution. While the error introduced by comparing colored solutions so widely different in strength might not be great, it was improbable that either the quality or intensity of the color would be the same under these different conditions of treatment. That this is the case is shown by the following experiment. Chloroform extracts of blood cholesterol obtained by the Autenrieth and Funk method[5] (Solutions A, B, F, and G, below) were compared with standards prepared (*a*) according to my directions, standard 0.5 mg. of cholesterol in 5 cc. of chloroform treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid; and (*b*) according to Weston's modification of my directions, standard 2.7 mg. of cholesterol in 10 cc. of chloroform treated with 4 cc. of acetic anhydride and 0.2 cc. of sulfuric acid. All were digested for 15 minutes in the dark at 22°C. The values, calculated in mg. per 100 cc. of blood, are shown in Table I.

The values obtained by Weston's modification are thus distinctly higher than those obtained by my directions. The colors were quite smoky in tint so that comparison was difficult. The high values may be in part due to the difference in tint, but probably more to the relatively low concentration of acetic

[4] Bloor, W. R., *J. Biol. Chem.*, 1916, xxv, 577.
[5] Autenrieth, W., and Funk, A., *Münch. med. Woch.*, 1913, lx, 1243.

TABLE I.

Solution.	By my directions.	By Weston's modification.
A	218	245
B	216	240
F	115	150
G	113	148

anhydride and sulfuric acid in Weston's standard which resulted in an incomplete development of the color.

Weston's experiment was repeated as follows:

Two samples of 50 cc. each of fresh beef plasma were taken and to one were added 5 cc. of ether containing 50 mg. of cholesterol, and to the other 5 cc. of ether alone (Weston did not add ether to his control serum). The two solutions were well shaken and after standing for about 2 hours with occasional shaking, samples were taken from them as follows.

a. Eight 3 cc. samples were taken from each and extracted with alcohol-ether according to directions in my method.[6] 10 cc. of the extracts were used for the determination.

b. Four 2 cc. samples were taken from each, digested with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk.[5] The chloroform extracts after dehydration and filtering were made up to 100 cc. 15 cc. samples were used for the determinations and were measured into small beakers, evaporated to less than 5 cc., cooled, transferred to the 10 cc. cylinders, and made up to 5 cc. with the washings from the beakers.

c. Four 50 cc. samples of the alcohol-ether extracts from *a* were evaporated almost to dryness, then treated with alkali, and extracted with chloroform according to the directions of Autenrieth and Funk, using corresponding amounts of alkali and chloroform.

In all determinations the samples and standard were each contained in 5 cc. of chloroform and were treated with 2 cc. of acetic anhydride and 0.1 cc. of concentrated sulfuric acid in the dark at room temperature (20–22°C.) for 15 minutes. The Duboscq colorimeter was used throughout and there can be no question that it is more accurate and less subject to personal error than the Autenrieth-Königsberger instrument, certainly than the "*Hellige*" form which appears in this country. The Duboscq has also the advantage that the standard and test solutions are of about the same strength. It may be noted here that Autenrieth and Funk did not use a cholesterol standard, but instead used a permanent standard made of a mixture of metallic salts which was calibrated by the use of known cholesterol solutions, thus avoiding the difficulty mentioned

[6] Bloor, *J. Biol. Chem.*, 1916, xxiv, 227.

above. A permanent standard such as Autenrieth and Funk used has some advantages especially for use with the Autenrieth-Königsberger type of colorimeter, and as they do not give the composition of their solution, an attempt is being made to prepare a suitable solution.

The results of single consecutive determinations on these samples are given in Table II. The values are expressed in mg. per 100 cc. of blood.

TABLE II.

A. My procedure.		B. Autenrieth and Funk's procedure.	
Sample.	Value.	Directly on blood.	
		Sample.	Value.
Control plasma.		Control plasma.	
1	130	A	114
2	130	B	115
3	130	C	118
4	128	D	118
5	128	Plasma plus 1 mg. of cholesterol per 1 cc.	
6	129	E	218
7	129	F	216
8	129	G	218
		H	216
		On alcohol-ether extracts.	
Plasma plus 1 mg. of cholesterol per 1 cc.		Control.	
		1	119
		Plasma plus cholesterol.	
1	227	1	219
2	229	2	214
3	229	3	219
4	230		
5	229		
6	235		
7	235		
8	229		

These results show that the method will do all that has been claimed for it when used as originally directed. Satisfactory recovery of added cholesterol is obtained either by the use of

my method throughout or by the application of my procedure for color production to extracts obtained by the Autenrieth-Funk digestion either of plasma direct or of alcohol-ether extracts of it.

The alkali treatment either of plasma or of alcohol-ether extracts of it results in lower values.

The points enumerated below are sufficient to explain the low values reported by Weston but not the very high ones obtained by him in some cases. My method of cholesterol determination is based on the Liebermann-Burchard color reaction as used by Autenrieth and Funk, adapted for use with the alcohol-ether extraction of blood. The only departure from Autenrieth and Funk's directions (aside from the use of a cholesterol standard instead of a salt standard) is that the color production is carried out at room temperature instead of at about 32°C. (obtained by setting the color tubes in a beaker of water at 32–35°—in which the temperature may be 3° or 4° lower by the end of the 15 minutes). The change to room temperature was made as the result of experiments which showed that slightly higher results were obtained at the lower temperature and that the solutions were free from a yellowish tint which developed at the higher temperature. In the published directions no definite temperature was specified and as this seemed to be a possible source of error, temperature conditions were sought other than those in which the reaction was ordinarily carried out. Ordinarily the cylinders in which the color was produced were placed in the cupboard of a table in the middle of the laboratory where the temperature was at least as constant as that of the laboratory which is kept at 20–23°C. for all but the hottest months of the year. On the other hand, the temperature in a partly enclosed window-seat of a north window in the laboratory was found to be 12°C. A series of samples were therefore allowed to digest 15 minutes in a dark box there. The values obtained for blood cholesterol were very high, almost double those obtained at 21°C., and were quite irregular. The color in the standard cholesterol cylinder was obviously only little developed while that in the blood cholesterol cylinder was well developed. These results indicated that the standard cholesterol did not react at the same rate as the blood cholesterol, and other experiments to test this

point were carried out as follows. Solution A was a chloroform extract of residues from evaporation of mixed alcohol-ether extracts of human blood. Solution B was a standard cholesterol (Kahlbaum) solution in chloroform containing 0.5 mg. of cholesterol in 5 cc. Pairs of samples consisting of one each of these solutions were digested at temperatures of 10–34° as follows.

5 cc. of each solution were measured into a 10 cc. flask and 2 cc. of acetic anhydride added. The solution was adjusted to the required temperature by shaking in water at that temperature, 0.1 cc. of concentrated sulfuric acid added, the temperature again quickly adjusted, and the little flasks were placed in water in a beaker thermostat at the desired temperature, covered, and set in the dark for 15 minutes. At the end of that time the reaction was stopped by chilling the solutions in ice water and comparisons were made at once with the standard solution.

As a standard for each of the determinations a 5 cc. sample of the standard cholesterol was digested at 22° in the same way as the test samples, then chilled before use. The results of a typical series are given in Table III.

TABLE III.

Temperature.	Solution A (blood cholesterol).	Tint* of color.	Solution B (Kahlbaum's cholesterol).	Tint* of color.
°C.	mg.		mg.	
10	0.48±**	Blue.	Trace.	
14	0.62	Bluish.	0.21±**	Blue.
18	0.62	"	0.30±**	"
22	0.68	Matched.	0.50	Matched.
26	0.62	Faint yellowish.	0.52	Faint yellowish.
30	0.60±**	Yellow.	0.48	Yellowish.
34	0.58±**	Deep yellow.	0.40±**	Yellow.

* As compared with the standard.

** These values are approximate because of the difficulty of comparing colors of different tints.

Extracts from other blood samples showed some variations from the above, but in general the results were the same and indicate that the blood cholesterol behaves quite differently from the standard cholesterol. It reacts more readily with the re-

agents, comes to its maximum sooner, and begins to fade sooner than the standard (gall-stone?) cholesterol. Whether the blood cholesterol is wholly or in part a different substance from the standard or whether the difference in speed of reaction is due to an impurity in the blood extracts which accelerates the reaction is not clear, but in view of these differences in rate a further study of the Liebermann-Burchard reaction in its application to blood cholesterol is desirable and is now being undertaken in this laboratory.

The results obtained above indicate that values obtained at 22° are more nearly correct than those obtained at either higher or lower temperatures. At lower temperatures the standard color has not fully developed while at the higher temperatures the blood cholesterol color has passed its maximum and has begun to fade.

The experiments recorded in Table III throw an interesting light on the question of tints of color in blood cholesterol determinations. Mueller[2] and Weston[1] have both reported yellowish or brownish tints in the colors obtained from extracts made by my method. As noted above, the color, whether of blood cholesterol or of the standard cholesterol, passes through the stages of blue-green, green, yellow-green, and finally yellow, and whether the standard solution has a bluish or yellowish tint as compared with the test solution depends on the relative stage of development of the two. There is, of course, a considerable personal factor in judging tints of color, but in the course of a great number of blood cholesterol determinations carried out by my method at room temperature (22°) I have found very few in which the difference of tint was sufficient to make any difficulty in reading. In some cases, most frequently in extracts of whole blood, the alcohol-ether extract was brown or brownish green, and it was found that this color carried through into the chloroform. To get rid of this color Mueller's[2] directions have been found useful. After evaporating the alcohol-ether extract and extracting the residue with chloroform (using about 15 cc.), the chloroform is washed with water, dried with anhydrous sodium sulfate, evaporated to small volume, and the color produced in the cylinders as usual. The brownish color is soluble in water and is almost entirely removed by this treatment. A simpler way of

accomplishing the same result (due to Dr. W. E. McEllroy of this laboratory) is to shake the alcohol-ether extract with about 5 gm. of the anhydrous sulfate. Occasionally a sample is obtained (I have had three) in which the alcohol-ether extract is colorless but which develops a brown color in the chloroform on treatment with acetic anhydride and sulfuric acid. Washing or treatment with sodium sulfate was less effective in these cases. Some form of alkali treatment was necessary either with sodium ethylate, as was originally directed,[7] or with strong watery alkali and extraction, as in Autenrieth and Funk's method.

SUMMARY.

Results reported by Weston in the use of my method for the determination of cholesterol in blood are discussed and probable reasons for their inconsistency noted.

Differences in behavior of blood cholesterol and standard (Kahlbaum's) cholesterol are found.

A temperature of 22°C. has been found most suitable for the color production.

Causes of "off color" are discussed and suggestions offered for their correction.

[7] Bloor, *J. Biol. Chem.*, 1915, xxiii, 317.

A NOTE ON THE DIURNAL VARIATIONS IN CREATINE EXCRETION.

BY W. DENIS.

WITH THE ASSISTANCE OF ANNA S. MINOT.

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(Received for publication, January 31, 1917.)

A short time ago during the course of a series of observations on the urine of a woman suffering from Graves' disease, we made the discovery that while the day urine of this patient contained considerable quantities of creatine, the urine passed during the night was practically creatine-free.

This observation has been followed by creatine determinations in the day and night urines of a number of hospital patients, with the result that in every case examined it has been found that the excretion of creatine is in a large measure confined to the hours between 7 a.m. and 7 p.m. The results of our observations are given in the tables.

The experimental subjects were all patients at the Massachusetts General Hospital. They were kept in bed during the period of observation and were fed during this period and 2 days previously a liberal diet free from meat, fish, peas, or beans.

The creatinine and creatine determinations were made by Folin's[1] micro methods. Picric acid carefully purified by the method described by Folin and Doisy[2] was used throughout the work.

In Table I are presented the results obtained by collecting the urine in 12 hour periods from 7 a.m. to 7 p.m. and from 7 p.m. to 7 a.m.

As will be seen, the quantity of creatine excreted during the night is but a fraction of that obtained in the day urine, while

[1] Folin, O., *J. Biol. Chem.*, 1914, xvii, 472.

[2] Folin, O., and Doisy, E. A., *J. Biol. Chem.*, 1916–17, xxviii, 349.

TABLE I.

No.	Sex.	Age.	Diagnosis.	Day. 7 a.m.—7 p.m. Volume.	Day. Preformed creatinine.	Day. Creatine.	Night. 7 p.m.—7 a.m. Volume.	Night. Preformed creatinine.	Night. Creatine.
		yrs.		cc.	gm.	gm.	cc.	gm.	gm.
1	♀	24	Graves' disease, mild case	560	0.32	0.15	500	0.30	0.02
2	♀	27	Graves' disease, mild case	1,500	0.32	0.21	820	0.37	0
3	♀	32	Graves' disease	1,100	0.37	0.26	505	0.31	0.08
4			Same patient	900	0.36	0.23	610	0.33	0.03
5	♂	65	Cirrhosis of liver, ascites	905	0.67	0.19	640	0.60	0
6	♂	44	Pneumonia, temperature 100–100.4° F.	650	0.71	0.23	660	0.70	0.02
7	♀	19	Graves' disease, very severe case	610	0.30	0.26	700	0.30	0.06
8	♂	10		860	0.18	0.16	665	0:17	0.03
9	♂	10	Nephritis	860	0.28	0.07	400	0.25	0.01

in some cases creatine is entirely absent from the night urine, while present in considerable quantities in that passed during the day.

In order to study to greater advantage the effect, if any, produced by the ingestion of food on the creatine output, we have

TABLE II.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	mg.	mg.	mg.	
7– 9 a.m.	90	810	51	20	Female, 24 yrs. old, weight 54.5 kg. Graves' disease.
9–11 "	120	1,010	52	32	Breakfast at 8.
11 a.m.–1 p.m.	200	925	50	10	Dinner at 12.
1–3 p.m.	175	1,100	60	44	
3–5 "	160	960	52	20	
5–7 "	210	1,186	54	32	Supper at 5.
7 p.m.–7 a.m.	440	2,422	290	30	

Total creatine excretion for the day (12 hrs.) 158 mg.
" " " " " night (12 ") 30 "

made 2 hour collections from 7 a.m. to 7 p.m. in five cases. The results of these observations are presented in Tables II to VI inclusive.

After the above work had been completed our attention was called to a paper by Powis and Raper[3] which we had overlooked. These investigators studied the diurnal and nocturnal variations in the

TABLE III.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	mg.	mg.	mg.	
7- 9 a.m.	156	672	56	70	Female, 20 yrs. old, weight 50 kg. Graves' disease, severe case.
9-11 "	210	900	52	102	Breakfast at 8.15.
11 a.m.-1 p.m.	320	1,185	60	84	Dinner at 12.20.
1-3 p.m.	275	1,100	60	42	
3-5 "	220	762	56	44	
5-7 "	190	505	55	52	Supper at 5.15.
7 p.m.-7 a.m.	430		360	60	

Total creatine excretion for the day (12 hrs.) 394 mg.
" " " " " night (12 ") 60 "

TABLE IV.

Time.	Volume.	Preformed creatinine.	Creatine.	Remarks.
	cc.	mg.	mg.	
7-11 a.m.	276	130	80	Female, 32 yrs. old, weight 68.1 kg. Graves' disease.
11 a.m.-1 p.m.	225	55	42	Breakfast at 7.45.
1-3 p.m.	475	70	80	Dinner at 12.20.
3-5 "	220	70	22	
5-7 "	370	70	41	Supper at 5.
7 p.m.-7 a.m.	700	420	110	

Total creatine excretion for the day (12 hrs.) 265 mg.
" " " " " night (12 ") 110 "

[3] Powis, F., and Raper, H. S., *Biochem. J.*, 1916, x, 363.

TABLE V.

Time.	Volume.	Total nitrogen.	Preformed creatinine.	Creatine.	Remarks.
	cc.	mg.	mg.	mg.	
7– 9 a.m.	150	422	30	20	Boy, 10 yrs. old, convalescent after amputation of leg.
9–11 "	170	530	34	41	Breakfast at 8.15.
11 a.m.–1 p.m.	110	490	29	18	Dinner at 12.30.
1–5 p.m.	415	1,330	65	39	
5–7 "	122	692	29	10	Supper at 5.
7 p.m.–7 a.m.	400	2,925	173	28	

Total creatine excretion for the day (12 hrs.) 128 mg.
" " " " " night (12 ") 28 "

TABLE VI.

Time.	Volume.	Preformed creatinine.	Creatine.	Remarks.
	cc.	mg.	mg.	
7– 9 a.m.	100	30	20	Boy, 12 yrs. old, weight 31.8 kg. Convalescent from nephritis.
9–11 "	150	33	10	Breakfast at 8.10.
11 a.m.–1 p.m.	210	29	11	Dinner at 12.30.
1–5 p.m.	410	70	41	Supper at 5.
5–7 "	100	27	15	
7 p.m.–7 a.m.	250	180	0	

Total creatine excretion for the day (12 hrs.) 97 mg.
" " " " " night (12 ") 0 "

excretion of creatine by a normal 3 year old child. They found that in this subject the rate of creatine excretion was highest during the morning, decreased somewhat in the later afternoon, and was very small during the night. These variations in creatine excretion are, according to Powis and Raper, not associated with the ingestion of food, but are due to the fact that the body when at rest has greater power to utilize creatine than when the muscles are in action.

As will be seen from our results the excretion of creatine by

persons on a strictly creatine-free diet varies from hour to hour; in most of the cases examined it attained a maximum in about 2 hours after the substantial meal of the day. Thus the subject used in Table I ate but little breakfast (a small dish of oatmeal, one slice of toast, and one glass of milk), but partook of a substantial dinner at noon (tomato purée, potato, two eggs, bread, milk, ice-cream); it will be noted that in her case the creatine excretion was not great during the morning hours, but attained a maximum between 1 and 3 o'clock in the afternoon. On the other hand the subject used in Table IV ate large quantities of food both at breakfast and dinner and shows a corresponding rise in creatine excretion after both of these meals.

It is also to be noted that on the whole the rise in nitrogen excretion following the ingestion of protein lags behind the curve of increased creatine excretion apparent after each meal.

Our results therefore do not confirm the finding of Powis and Raper as regards the lack of influence of food assimilation on creatine excretion. The source of urinary creatine, in spite of scores of investigations directed towards its elucidation, still remains obscure. The hypothesis that the creatine of the urine arises from the creatine of muscle is an inviting theory but one which as yet lacks indisputable proof. The acceptance of such a theory would place creatine among the metabolism products of endogenous origin. Our results make it seem highly probable, however, that creatine is of purely exogenous origin, and that its excretion is directly dependent on the intake of food.

DIRECT DETERMINATIONS OF PERMEABILITY.

By R. P. WODEHOUSE.

(Contributions from the Bermuda Biological Station for Research, No. 60, and from the Laboratory of Plant Physiology, Harvard University, Cambridge.)

(Received for publication, February 23, 1917.)

It is desirable to determine directly the penetration of substances into the living cell, but in most cases this is difficult or impossible. In order to make such direct determinations it is necessary to compare the composition of the cell sap with the composition of the liquid in which the cell is immersed. In the case of tissues this is impossible, because in crushing the cells to obtain the cell sap the latter is contaminated by admixtures of material from the intercellular spaces as well as altered by chemical action and adsorption. It is therefore necessary to employ individual cells and to extract their cell sap without opportunity for contamination or change.

Most cells are too small to yield satisfactory results by this method. The writer, however, has been fortunate in having an opportunity to experiment with the marine alga *Valonia*, which has individual cells so large that it is easy to get from a single cell enough sap upon which to make chemical tests. Cells of the average size yield from 1 to 2 cc. of sap, and it is not unusual to obtain cells which yield as much as 5 cc.

The cells were removed from the sea water, rinsed in distilled water, dried with filter paper, and then punctured with a needle. On pressing the cell wall the sap squirted out and was collected. This sap is contained in the large central vacuole of the cell (or cœnocyte).

Upon examination the cell sap was found to differ, in most cases, materially from sea water. The sap was taken from a few plants and tested qualitatively for the presence of some of the salts of sea water.

As for the metal ions of sea water, sodium could easily be demonstrated within the cell by allowing some of the sap to evaporate on a watch-glass when crystals of sodium chloride were seen to be formed. The presence of calcium was easily demonstrated. When a saturated solution of ammonium oxalate was added to the cell sap a white precipitate was produced which was soluble in hydrochloric acid but insoluble in acetic acid This precipitate was washed in acetic acid and water and when dissolved in a small amount of hydrochloric acid gave an unmistakable flame test for calcium.

A similar condition, however, was not found to hold for magnesium. In sea water this can easily be demonstrated by precipitating with any of the fixed alkali hydroxides (*e.g.*, NaOH) forming $Mg(OH)_2$, which is white, gelatinous, and insoluble in excess of the reagent but readily soluble in the presence of ammonium salts, and from such solution can readily be precipitated by the alkali phosphates (*e.g.*, Na_2HPO_4) in the form of magnesium ammonium phosphate. When this method was tried on the cell sap, however, only the merest trace of a precipitate was obtained by the addition of NaOH and, though this disappeared upon the addition of ammonium sulfate, the addition of disodium phosphate to the solution would not cause the precipitation of any magnesium ammonium phosphate. This shows that, though magnesium is probably present within the cell, it does not exist in nearly as high a concentration as in sea water.

Exactly the opposite obtains in the case of potassium. In sea water the concentration of potassium is so low that no precipitate was produced by the addition of picric acid, and the dried residue gave only a doubtful flame test. With the *Valonia* sap, however, the dried residue showed an abundance of potassium by the flame test; when picric acid was added to the sap long acicular crystals settled out, and when these were dried and touched with a hot needle they detonated, giving an unmistakable potassium-colored flash, showing that these crystals, formed by the addition of picric acid, were potassium picrate. From this it is plain that there is a considerably higher concentration of potassium within the cell than without.

The *Valonia* sap gives an abundant precipitate with chloroplatinic acid. The solution was heated to complete the reaction

and when allowed to cool, the precipitate formed at room temperature and could be only slightly increased in amount by cooling to zero. Upon examination with the microscope this precipitate was found to consist mostly of octahedral crystals, the rest being made up of crystals of other forms but obviously of the same material because similar crystal forms were found in the precipitate in the control test with chemically pure potassium oxalate. This test seems to me to be conclusive for the presence of potassium. Its abundance in the sap is shown by a control test done on the Bermuda sea water. Keeping everything exactly the same it was impossible to get the potassium chloroplatinate crystals to form even by cooling down to zero. Small crystals could be caused to form only by evaporating the mixture nearly to dryness and adding several volumes of absolute alcohol. The conclusion arrived at from the picric acid test is thus confirmed.

The proportions of the four kations Na, Ca, Mg, and K, could not be determined quantitatively on account of the limited amount of material available at the time when the work was done. This is very important from the viewpoint of antagonism and the author hopes to make it the subject of further investigation.

When the anions of sea water were tested for the presence of chlorides they were indicated in all the tests made by the copious precipitate produced by the sap with silver nitrate. Barium chloride, however, failed to give any precipitate with the sap, thus showing the absence of sulfates. Nevertheless, barium chloride gave an abundant precipitate with the sea water from which the plants had been taken. Occasionally a cell was found which showed the presence of sulfate in the cell sap by the barium chloride test. Upon further investigation it was found that cells which were obviously dead always showed the presence of sulfate and that those which appeared to be in an unhealthy condition frequently gave a positive test. The cells which seemed to be healthy rarely gave any precipitate at all with barium chloride. Accordingly the following series of tests was made to determine if the presence of sulfate was associated with death or injury of the cell. On holding the cells up to the light the healthy ones were seen to have a uniform green color

over the entire surface, while cells which are described below as in poor condition had a mottled appearance, or the protoplasm appeared to be slightly shrunken, or else the cells, when held between the thumb and finger, could be felt to lack the normal turgidity.

In each case the description of the cell was written down before the test was made, in order to avoid subjective error.

No.	Description of cell.	$BaCl_2$ test.
1	Largest cell found (over 1 inch long).	No ppt.
2	Large, dead; protoplasm dead and shrunken.	Ppt.
3	Almost as large as No. 1; in good condition; old.	No ppt.
4	Small, average size, apparently healthy.	"
5	" " " " "	"
6	" " " " "	"
7	" " " " "	"
8	Large, but healthy.	"
9	Large; not in good condition but apparently still alive.	Ppt.
10	Appears to be in poor condition, not quite turgid but apparently still alive.	"
11	Much like No. 10.	No ppt.
12	Average cell in good condition.	"
13	" " " " "	"
14	Large and old but in good condition.	"
15	Small cell apparently healthy.	"
16	" " " "	Ppt.
17	Average cell in good condition.	No ppt.
18	An old cell but apparently healthy.	"
19	Normal, average size.	"

Besides these cells, which were taken as nearly as possible at random, thirty-six other cells were chosen as being undoubtedly healthy, and of these only one showed the presence of sulfate. From experience it is possible, by inspection, to be almost always, though never absolutely, certain which cells are going to show the presence of sulfate.

From this it seems evident that sap of live and healthy *Valonia* cells does not contain free sulfate in large enough quantities to give a visible precipitate with barium chloride. It is also equally certain that when the cell dies sulfate enters, and it follows from this that the presence of sulfate in the cell indicates injury or death, even though no other manifestations are visible.

In order to preclude the possibility that sulfate was present, but in some chemical or physical union which prevented it from reacting with barium chloride, *Valonia* sap which had been shown to give a negative test with this reagent was boiled with nitric acid of several different concentrations, in order to break up any such possible union, and tested again. In each case, however, the test for sulfate was negative.

In order to test the matter still further, some of the sap was incinerated in a porcelain crucible. Upon incineration it showed the presence of a great deal of organic material, which left a deposit of carbon, but upon being boiled with HCl and incinerated again this was removed and the residue was completely soluble in water. The sea water, on the other hand, showed no organic material and proved to be largely insoluble in water after the same treatment. The soluble part, however, gave a copious white precipitate with barium chloride, while the *Valonia* sap after incineration with HCl gave just a trace, scarcely enough to be visible.

The conclusion from the above experiment is that there is little or no sulfate within the vacuole of the normal *Valonia* cell. Consequently, since there is an abundance of sulfate in the surrounding sea water, the semipermeable protoplasmic membrane must possess a selective permeability which renders it impermeable to sulfate as long as it is alive. This, however, is lost immediately upon death.

It is of course possible that sulfate may penetrate the outer plasma membrane into the protoplasm without being able to penetrate the vacuole membrane into the vacuole, but upon this point the experiment throws no light.

In order to test for permeability to nitrates, some cells were placed in sea water to which enough potassium nitrate had been added to give a good nitron test. After 24 hours the cells were still alive (they remained alive for several days when replaced in sea water) and showed the presence of nitrate within by the nitron test. The same was true of sodium nitrate. But it appeared upon examination that the control cells in normal sea water also showed the presence of nitrates by the nitron test. Sea water did not show the presence of nitrate by the nitron test. It is therefore evident that the nitrates within the cell must have

penetrated through the plasma membrane, but this may have taken place wholly or in part before the nitrates were added to the sea water.

The mechanism by which nitrates and potassium are stored within the cell so as to remain at a higher concentration than in the surrounding sea water deserves further study.

It is of interest that the cell wall acts as an osmotic membrane but possesses no selective permeability. Dead cells are often turgid even though the protoplasm has shrunken away from the cell wall. These cells when put into a hypertonic salt solution almost immediately become flaccid, and when replaced in sea water or in fresh water they again become turgid. However, if dead cells are placed in water containing nitrates, nitrate can be demonstrated within them shortly afterwards. If living cells are killed and then replaced in sea water, sulfate can soon be shown to be present inside. This shows that salts can pass more or less freely through the cell wall and yet it offers sufficient obstruction to enable it to act as an osmotic membrane.

The writer takes pleasure in expressing his thanks to Professor E. L. Mark for the use of the facilities of the Bermuda Biological Station, where this investigation was made, to Professor W. J. V. Osterhout for his interest in the problem and many helpful suggestions, and to an unknown friend who assisted in bearing the expenses of the Bermuda trip.

THE MICRO-TITRATION OF AMMONIA, WITH SOME OBSERVATIONS ON NORMAL HUMAN BLOOD.

By GEORGE D. BARNETT.

(*From the Laboratory of the Medical Division of the Stanford University Medical School, San Francisco.*)

(Received for publication, February 3, 1917.)

In connection with the study of the ammonia poisoning which occurs in rabbits following the administration of large doses of urea, the problem of determining ammonia in small quantities of body fluids has recently come up in this laboratory and certain refinements in the micro-titration of ammonia have been introduced. Our procedure differs from the usual aeration-titration method only in the use of an accurate microburette, 0.005 N alkali, and in a few details of titration, but it seems to offer certain advantages where the total quantity of ammonia available is very small, as in blood and tissue ammonia determinations, and in serial determinations of blood urea in small laboratory animals.

Method.

10 cc. of the solution to be investigated (containing less than 0.75 mg. of ammonia per 100 cc.) are placed in a large test-tube (22 x 380 mm.) and 10 cc. of saturated potassium carbonate solution added. The trace of ammonia present in the potassium carbonate solution should be removed by prolonged aeration of the stock bottle. Even with test-tubes of this depth a very small amount of alkaline "spray" will pass over into the absorption tube, but this can be prevented by interposing a short tube containing a loose plug of cotton, as suggested by Folin.[1] For absorption tubes we use still larger test-tubes (30 x 410 mm.) containing an accurately measured volume (less than 0.5 cc.) of 0.01 N hydrochloric acid in about 15 cc. of distilled water. This

[1] Folin, O., *Z. physiol. Chem.*, 1902–03, xxxvii, 161.

acid is measured from an automatic capillary pipette connected with a small stock bottle. If kept in seasoned glass the deterioration of this weak acid (and also of the alkali) is inappreciable over a period of a few weeks. The advantage in the use of deep absorption tubes is the entire absence of loss by spattering, even with vigorous aeration. An aeration rate of 5 liters per minute for 20 minutes is satisfactory. We have found it of advantage in maintaining a steady air current to use a simple water bottle manometer calibrated in liters per minute by the method of Kober and Graves,[2] and connected to the distal end of the system which is being aerated.

If the absorption tubes are not of Jena or some "non-soluble" glass, a slight correction must be made for the alkali dissolved during the aeration. This correction is readily determined by passing the same air current through a sample of acid, and is constant for a given technique. In our tubes, and with the technique here given it amounts to 0.01 cc. of 0.005 N acid.

Titration of the excess acid is carried out in the absorption tube without removal of the aeration tube, a gentle current of air being passed through the acid for the purpose of stirring. 0.005 N alkali is run in from a 1 cc. microburette, made from a 1 cc. pipette graduated in hundredths and accurately calibrated. The tip of the burette is drawn out to deliver a drop of 0.01 cc. or less. Using methyl red as an indicator (four drops of a saturated solution in 70 per cent alcohol) a sharp end-point is readily obtained with half a drop (0.005 cc.) of the 0.005 N alkali. A fixed end-point is used for comparison, and sharper results are obtained if the color of the end-point is chosen where the pink color of the indicator just begins to show traces of yellow. For a fixed end-point we use a sodium acetate-acetic acid mixture. It is also of extreme importance to avoid the use of water containing carbon dioxide. Water from our laboratory Stokes still cannot be used at all unless boiled or redistilled over lime.

When the end-point is neared the stirring tube is removed, being carefully washed down by means of water from a bulb wash bottle; not from a bottle blown by mouth, on account of the carbon dioxide, nor from a shelf bottle, on account of

[2] Kober, P. A., and Graves, S. A., *J. Am. Chem. Soc.*, 1913, xxxv, 1594.

the alkali of the rubber tubing. The inside of the titration tube is then washed down, the volume made up to 25 cc., or any convenient small end-volume, as long as all samples are the same, and the titration completed, adding the alkali in successive amounts of 0.005 cc. and shaking or stirring gently before comparison with the fixed end-point.

Ten consecutive analyses of a solution of ammonium sulfate containing 0.12 mg. of NH_3 per 100 cc. gave results as follows:

1.	0.12	6.	0.12
2.	0.12	7.	0.11
3.	0.12	8.	0.12
4.	0.12	9.	0.11
5.	0.11	10.	0.12

We have applied the method to the determination of ammonia in oxalated human blood in a few normal cases. Since Medwedew[3] in 1911 called attention to the rapid formation of ammonia in shed blood it has been recognized that the figures for blood ammonia given by earlier investigators are much too high. Probably none of the results represent the actual preformed ammonia present in the circulating blood, but are largely merely a measure of the extent to which the labile ammonia-yielding bodies of the blood have been disintegrated. The results least open to criticism in this regard are those of Rohde,[4] who with the vividiffusion method found in the dialysate in equilibrium with dog blood 0.18 mg. of ammonia nitrogen per 100 cc. (= 0.19 mg. of NH_3).

As far as human blood is concerned, no means of overcoming the difficulty has been devised, and the best we can do is to minimize the error from the ammonia increase by beginning the aeration as soon as possible after the blood is drawn, and completing the analysis as rapidly as possible. The aeration methods of Folin have greatly facilitated such determinations. The only figures we have found for normal human blood published since the importance of rapid methods has been realized are those of Gettler and Baker,[5] who in a series of thirty cases, using the

[3] Medwedew, A., *Z. physiol. Chem.*, 1911, lxxii, 410.
[4] Rohde, A., *J. Biol. Chem.*, 1915, xxi, 325.
[5] Gettler, A. O., and Baker, W., *J. Biol. Chem.*, 1916, xxv, 211.

method of Folin and Denis,[6] found 0.4 to 0.75 mg. of ammonia nitrogen per 100 cc. (= 0.42 to 0.79 mg. of NH_3). It will be noted that these figures are about ten times those given here. Our aeration has been begun within 1 to 2 minutes after drawing the blood. In order to emphasize the importance of this, figures obtained from aeration of samples of the same blood begun after 30 minutes are given. It will be seen that the ammonia obtained is two or three times that of the samples aerated immediately.

Ammonia Found in Oxalated Human Blood.

No.	Aeration begun within.	NH_3 per 100 cc.	Aeration begun within.	NH_3 per 100 cc.
	min.	*mg.*	*min.*	*mg.*
1	1	0.05		
2	2	0.00	30	0.07
3	2	0.04	30	0.15
4	1½	0.03	30	0.10
5	2½	0.04	30	0.08

SUMMARY.

1. Modifications of the aeration-titration method of determining ammonia are described, for use with small amounts of fluids of low ammonia content.

2. Figures are given for ammonia obtained from fresh oxalated blood in five normal individuals.

[6] Folin, O., and Denis, W., *J. Biol. Chem.*, 1912, xi, 532.

STUDIES ON CHOLESTEROL.

III. THE INFLUENCE OF BILE DERIVATIVES IN BLOOR'S CHOLESTEROL DETERMINATION.*

PRELIMINARY REPORT.

BY GEORGINE LUDEN.

(*From the Mayo Foundation for Medical Education and Research, Rochester, Minn.*)

(Received for publication, February 23, 1917.)

The main object of the following communication was to determine whether bile derivatives were capable of giving the Liebermann reaction in the absence of cholesterol, and whether their color reaction could be destroyed by the use of sodium ethylate under conditions similar to those in Bloor's cholesterol determination.

The origin of the "brownish tint," well known to all who have made blood cholesterol determinations, and the relative merits of colorimetric and gravimetric methods for the determination of cholesterol have been repeatedly discussed (Bloor (2, 3, 4), Mueller, Weston). It will be remembered that Bloor, in the first publication of his new method (2), specially mentions the addition of sodium ethylate to the ether-alcohol extract of the blood before evaporation, and that he left out the sodium ethylate in his modification of this method (3). For brevity, I shall refer to Bloor's original method as Bloor I and to its modification as Bloor II. The comments of Mueller on the Bloor method for cholesterol determination and Bloor and Knudson's reply (4) to these comments both refer to the Bloor II method only. Mueller's conclusions were that the values obtained by the Bloor method were increased by "other ether-alcohol- and chloroform-soluble substances" that were not cholesterol; that oxycholesterol might be among these substances; but that our knowledge of oxycholesterol is too indefinite to warrant positive conclusions. Bloor and Knudson claimed to show by a new procedure that there were either no other substances in the blood plasma, or that they behaved like ordinary cholesterol when treated with digitonin.

* Study I, *J. Lab. and Clin. Med.*, 1916, i, 662; Study II, *J. Biol. Chem.*, 1916, xxvii, 273.

stage could be observed in cholesterol solutions exceeding the concentration of standard tests from 10 to 400 times (Table I). Nor could the color sequence described by Windaus be made visible by using one-half or one-quarter of the amount of the reagents.

For brevity, the bile derivative solutions used in my experiments will be referred to as "pigment solutions" and the uniform solution with which the following tests were made as "Pigment 28 solution." The latter contained 0.2 mg. of bile derivatives in every 6 cc. of chloroform.

TABLE I.

*Color Reactions in Cholesterol Solutions.**

Test 1.—6 cc. of our stock solution, namely, 6 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—The colorless solution becomes dark green in a few seconds; no trace of the pink or the blue stage.

Test 2.—10 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—Identical with Test 1, except that the green color appears almost black.

Test 3.—15 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—The blue stage appears for a few seconds only and is blotted out by the density of the green stage. The solution appears black; its green tone can be recognized only in a very strong light.

Test 4.—200 mg. of cholesterol dissolved in 6 cc. of chloroform; usual reagents.

Reaction.—As the sulfuric acid is added, its progress towards the bottom of the test-tube is shown by a streak of purple-violet, but the next second the whole of the solution has turned black-green. Its green color can be recognized only near the top edge by shaking the test-tube.

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* The standard cholesterol solution used in all our blood cholesterol determinations contains 0.4 mg. of cholesterol in 6 cc. of chloroform. It is made up from a stock solution containing 200 mg. of cholesterol (Merck) in 200 cc. of chloroform.

Both standard and stock solutions are sealed with paraffin and kept on ice when not in use, in order to prevent any evaporation of the chloroform; the latter would increase their concentration, thereby affecting the accuracy of the tests.

Standardized graduated pipettes are used in all our tests in preference to graduated cylinders, since the latter often vary slightly and give less accurate results in consequence.

Apart from the color sequence referred to above, the pigment solutions showed the following peculiarities, which distinguished them from pure cholesterol solutions: (*a*) Their reaction was much slower than that of cholesterol; (*b*) the green stage of the reaction was olive-green, as compared with the emerald-green of pure cholesterol; (*c*) the green stage persisted unchanged from three to four times as long as the cholesterol green in solutions of equal strength; and (*d*) colorimetric determinations showed that the color value of the green stage was only a little over one-half of the cholesterol green in solutions of equal concentration, although, owing to the difference in tone, it appeared to be greater in the test-tube. In reference to these peculiarities the term "bile-green reaction" in the Liebermann test is tentatively suggested for the color reaction of these cholesterol-free bile derivatives.

A series of observations and systematic tests proved that the usual cholesterol standard test (0.4 mg. of cholesterol in 6 cc. of chloroform) reached its maximum color value at room temperature (20–22°C.) in 5 to 6 minutes, and maintained it for approximately 30 minutes; that the test lost one-third of its color value in 80 minutes and became colorless, *i.e.*, pale yellow, in 6 to 7 hours. On the other hand, pigment solutions of identical concentration reached their maximum color value in 190 minutes and remained unchanged for at least 24 hours. A record was kept of the exact time at which the reagents were added to the tests and revealed the length of the duration of the pink, blue, and green stages of the reaction in the pigment solutions at a room temperature of 20–22°C. (Table II). In every instance freshly made "ripe" standard tests not more than 20 minutes old were used for comparison. The advantage of making cholesterol determinations at room temperature rather than at a temperature of 35–37°C. has been discussed (11). Previous observations on the rapid reaction found in many pathologic blood samples have been fully corroborated by subsequent findings.

stage could be observed in cholesterol solutions exceeding the concentration of standard tests from 10 to 400 times (Table I). Nor could the color sequence described by Windaus be made visible by using one-half or one-quarter of the amount of the reagents.

For brevity, the bile derivative solutions used in my experiments will be referred to as "pigment solutions" and the uniform solution with which the following tests were made as "Pigment 28 solution." The latter contained 0.2 mg. of bile derivatives in every 6 cc. of chloroform.

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Test 2.—10 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—Identical with Test 1, except that the green color appears almost black.

Test 3.—15 mg. of cholesterol in 6 cc. of chloroform; usual reagents added.

Reaction.—The blue stage appears for a few seconds only and is blotted out by the density of the green stage. The solution appears black; its green tone can be recognized only in a very strong light.

Test 4.—200 mg. of cholesterol dissolved in 6 cc. of chloroform; usual reagents.

Reaction.—As the sulfuric acid is added, its progress towards the bottom of the test-tube is shown by a streak of purple-violet, but the next second the whole of the solution has turned black-green. Its green color can be recognized only near the top edge by shaking the test-tube.

* The standard cholesterol solution used in all our blood cholesterol determinations contains 0.4 mg. of cholesterol in 6 cc. of chloroform. It is made up from a stock solution containing 200 mg. of cholesterol (Merck) in 200 cc. of chloroform.

Both standard and stock solutions are sealed with paraffin and kept on ice when not in use, in order to prevent any evaporation of the chloroform; the latter would increase their concentration, thereby affecting the accuracy of the tests.

Standardized graduated pipettes are used in all our tests in preference to graduated cylinders, since the latter often vary slightly and give less accurate results in consequence.

TABLE II—*Concluded.*

Test 5.—0.040 mg. in 6 cc. of chloroform (three tests). (Standard cholesterol test 0.400 mg.)

Reaction.—Clear pale pink; then pale olive-green which lasts for about 3 hours.

Color Value.—Cannot be determined as the test looks gray in the colorimeter when compared with the emerald-green standard, although its color is clear pale green in the test-tube.

Test 6.—0.020 mg. in 6 cc. of chloroform (three tests). (Standard cholesterol test 0.400 mg.)

Reaction.—Faint but distinct pink; then very pale olive-green lasting about 1 hour.

Color Value.—Cannot be determined, for the same reasons as in Test 5. The green color is also clearly visible in the test-tube.

In order to study the effects of sodium ethylate on the cholesterol-free bile derivatives under conditions parallel to those found in the Bloor method, the following experiments were made:

The chloroform of a portion of the Pigment 28 solution was evaporated and the orange-colored residue extracted with ether-alcohol in the manner described by Bloor for blood samples. One portion of the ether-alcohol extract was treated according to the Bloor I method, with sodium ethylate, and the other according to the Bloor II method, without sodium ethylate. Overheating was carefully avoided during evaporation. After the usual extraction of the residue with chloroform, the Bloor I test was colorless and remained colorless when the usual reagents were added, even when the test was warmed. The Bloor II chloroform, on the contrary, was deep yellow and on the addition of the reagents gave the brilliant bile-green reaction already described. The experiment was repeated four times and gave identical results in every instance. Parallel tests made with pure cholesterol solutions treated in exactly the same way gave positive Liebermann reactions both with the Bloor I and the Bloor II methods. A slight loss of color value occurred in the pigment as well as in the cholesterol control tests. In the latter it was so small that it may be considered merely technical; 0.233 and 0.572 mg. of cholesterol were recovered when 0.240 and 0.600 mg. had been used respectively. In the former the loss was somewhat greater, but the factors which caused it cannot

be considered at present. The experiments showed conclusively, however, that the color reaction of cholesterol-free bile derivatives can be destroyed by sodium ethylate, whereas that of true cholesterol is not similarly affected. It should be added that the bile derivatives were readily soluble in ether, alcohol, chloroform, and petroleum ether (14), but that a precipitate was formed whenever sodium ethylate was added to the chloroform solution, whereas in the other solvents precipitation was barely perceptible. The bile-green type of Liebermann reaction could be obtained also with the usual reagents from the opaque residue left in the beakers used for the Bloor I tests after chloroform extraction by dissolving the residue in acid chloroform. These findings seem to support the deduction that the formation of a chloroform-insoluble sodium salt of bile derivatives may account for the difference observed in the Bloor I and Bloor II samples, and that the potassium salts of these substances may be more readily soluble in chloroform, especially in the presence of water. The occurrence of the brownish color in some of the Autenrieth tests and Mueller's[2] observations might also be explained thereby.

Although these experiments showed that the color reaction of bile derivatives obtained from gall-stones can be destroyed by sodium ethylate, it remained to be proved that closely allied or identical bodies were responsible for the high values found in icteric blood by the Bloor II method. The isolation of the bile derivatives in icteric blood proved extremely difficult, probably owing to the relatively small quantities in which they are present. Thirty attempts to isolate them were unsuccessful. It is possible that these failures may be explained by the observations of Hoover and Blankenhorn on "dissociated jaundice" and that several of the samples used in my experiments contained only bile acids. However, there may be other reasons connected with the

[2] The brownish color does not appear in every sample treated by the Autenrieth method, and I am unable to agree with Mueller's statement that it is "invariably present" in Bloor II tests. On the contrary, one-third of our determinations gave a brilliant green reaction that could be matched easily with the standard solution. Tests made by the Bloor I method always gave a perfect match.

various stages of oxidation described by Lifschütz (6–10) but these cannot be discussed at present.[3]

At last a simple method was found by which the bile derivatives of icteric blood could be isolated: 6 cc. of icteric blood were extracted with ether-alcohol according to the Bloor method. The extract was partly evaporated at room temperature. The remaining portion of the solvent, in which cholesterol crystals could be observed microscopically, was poured off. A black crust-like ring which had formed where the liquid receded during evaporation was dissolved in 2 per cent ammonia water, and the aqueous alkaline solution filtered, acidified, and shaken out with chloroform. The chloroform assumed a yellow tone and gave the bile-green type of Liebermann reaction. The bile derivatives, in this specimen of icteric blood at least, must therefore have been closely allied, if not identical with the cholesterol-free bile derivatives obtained from gall-stones. The difference between the Bloor I and Bloor II tests of this sample had been 0.280 mg. in the colorimetric determination for blood cholesterol.

A test devised by Lifschütz[4] for the differentiation between oxycholesterol and oxidized cholic acid proved of great value in establishing the identity of the bile derivatives in question. This test will be referred to as Lifschütz' "differential test." It is based on the relative solubility of oxycholesterol and oxidized cholic acid in chloroform and glacial acetic acid, and should not be confounded with the oxycholesterol color reaction. Lifschütz states that the oxycholesterol color reaction can be obtained alike in glacial acetic solutions of pure cholesterol, pure cholic acid, and ordinary bile after oxidation with benzoyl peroxide and the subsequent addition of eight drops of concentrated sulfuric acid and one drop of ferric perchloride (2 per cent solution in glacial acetic acid) to every 1 cc. portion of the test. If an equal volume of chloroform is added to the glacial acetic solutions, the green color of the oxidized cholesterol will be found in its entirety in the upper layer, the bottom layer being practically

[3] Because of Lifschütz' observations on the decomposition of cholesterol to bile acids, and Schulze and Winterstein's studies on the influence of the light on cholesterol, my pigment solutions have always been kept in the dark.

[4] Lifschütz (9), p. 346.

colorless (pale yellow or brownish), whereas if the test is made with oxidized cholic acid the colors will be reversed, the upper stratum will remain colorless after separation, and the bottom (chloroform) layer will contain all the green color. When bile is used, both layers will be colored in direct proportion to the amount of either substance present in each layer; the quantity can then be determined only spectroscopically, by means of the characteristic spectra. In the absence of spectroscopic facilities, however, oxycholesterol and oxidized cholic acid can be differentiated accurately by means of the differential test when only one substance is present in the solution. Lifschütz adds that the proportions he recommends must be strictly observed in order to obtain the above results. These proportions have therefore been used in the following experiments.

The chloroform component of (*a*) a cholesterol solution, (*b*) a portion of the Pigment 28 solution, and (*c*) a solution containing the cholesterol-free bile derivatives of icteric blood, was removed by evaporation and the residues were dissolved in glacial acetic acid. The glacial acetic solutions were treated in the manner described by Lifschütz and the differential test was made by adding an equal volume of chloroform. After separation had taken place the green color of the oxidized cholesterol was found in the upper layer, while the bottom layer had a faint pinkish brown tinge. In the two tests made with the bile derivatives, on the contrary, the upper layers were colorless and the green color could be seen in its entirety in the lower (chloroform) layers. Whether the differential test was made at the red, the blue, the green, or the terminal dirty brown stage of the reaction, the color component of the oxidized cholesterol was found in the upper stratum, whereas that of the bile derivatives invariably settled in the bottom layer. Moreover, the green stage of the bile derivatives remained unchanged for days, while the oxycholesterol green changed to dirty brown in about 12 hours. The latter peculiarity alone, which is in accordance with the color persistency of the bile-green reaction in my pigment solutions, might suffice to distinguish the bile derivatives from oxycholesterol, and recalls Lifschütz' statement concerning the color reaction of oxidized cholic acid, that colors like spectra remain for weeks. Although these tests showed that the bile

derivatives contained no oxycholesterol, but substances that were closely allied to cholic acid, a slight difference could be observed between the derivatives from gall-stones and those from icteric blood. The latter needed the complete oxidation required by cholic acid, while the former gave the Lifschütz reaction after oxidation with benzoyl peroxide alone, without the addition of sulfuric acid and ferric perchloride. It will be remembered that the color sequence of the Lifschütz reaction is the reverse of that seen in bile pigments under the influence of oxidizing agents.[5] The gall-stone derivatives must therefore have been partially oxidized. It is hard to understand where this partial oxidation could have occurred as no oxidizing agents except the atmospheric oxygen during extraction of the cholesterol had been used until Lifschütz' differential test was made. The following explanation is tentatively suggested: As bilirubin, a hemoglobin derivative (Mathews), could be demonstrated in the mixture by Hammarsten's test, and as Lifschütz (10) was able to prove the strongly oxidizing properties of hemoglobin, it seems possible that some of the bile pigments present in the mixture may have played a part in the partial oxidation of the cholic acid which it contained. The bile derivatives from icteric blood exhibited the following peculiarity: Although the upper layer remained colorless in Lifschütz' differential test, the colored bottom layer was composed of two strata, the lower and narrower being dark green, the other bright yellow with a green tinge. This showed that the bile derivatives did not contain oxidized cholic acid alone, but another closely allied substance as well. The latter may have been the "rhizocholic acid" mentioned by Mathews[6] or the "resin acid" described by St. Minovici and Zenovici.

Further investigations concerning the solubility of the bile salts, spectroscopic determinations, and tests with Rosenheim's new reaction for oxycholesterol will be reported when completed.

SUMMARY.

1. In 748 parallel blood cholesterol determinations by Bloor's original method with sodium ethylate (Bloor I) and its modification without sodium ethylate (Bloor II), a constant difference

[5] Von Reinbold (15), p. 278.

[6] Mathews (12), p. 430.

was observed in the cholesterol values obtained, lower values being registered by the Bloor I method.

2. In normal blood a constant slight difference (0.050 to 0.070 mg.) occurs between the values obtained by the Bloor I and Bloor II methods; in icteric blood differences of 0.090 to 0.280 mg. occur.

3. Since the Liebermann reaction could be obtained with a mixture of cholesterol-free gall-stone derivatives, the difference appears to be due to a combination of bile pigments and bile acids present in the blood.

4. The Liebermann reaction of cholesterol-free gall-stone derivatives differs from that of cholesterol inasmuch as the pink stage of the reaction is distinctly visible in weak solutions of the former, although it cannot be seen in cholesterol solutions of equal or up to 400 times greater concentration.

5. The type of Liebermann reaction given by gall-stone derivatives can be obtained from the cholesterol-free residue of icteric blood.

6. This color reaction is destroyed by the use of sodium ethylate under conditions parallel to those found in the Bloor I method. The color reaction of cholesterol is not affected by the use of sodium ethylate.

7. That the Liebermann reaction of cholesterol-free bile derivatives is due, not to oxycholesterol, but to cholic acid and allied substances in the presence of bile pigments, could be proved by Lifschütz' differential test.

8. Cholesterol-free gall-stone derivatives require less vigorous oxidation than bile derivatives from icteric blood in order to give Lifschütz' differential test.

9. The oxidizing properties of hemoglobin having been demonstrated by Lifschütz, and bilirubin, a hemoglobin derivative, being demonstrable in the gall-stone derivatives, it is tentatively suggested that the bile pigments contained in the latter may have caused their partial oxidation.

10. The experiments suggest that parallel determinations with Bloor's original method and its modification may furnish valuable information concerning the chemical constituents of the blood in cases of biliary disturbance with and without icterus, which might be supplemented by the dialyzation method of Hoover and Blankenhorn.

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THE HOURLY ELIMINATION OF CERTAIN URINARY CONSTITUENTS DURING BRIEF FASTS.

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The data presented in the present paper form the first part of a contemplated study of the influence of various agents (chiefly foodstuffs) upon the elimination of certain urinary constituents, with special reference to uric acid. As originally planned the work was to be essentially a repetition of the recent work of Mendel and Stehle,[1] making use of the microcolorimetric method for uric acid determinations, instead of the Folin-Shaffer[2] method, which Mendel and Stehle employed. We believe that the Folin-Shaffer method is not well adapted to the study of *hourly* uric acid elimination,[3] especially where the output falls below 10 mg. or the volume of urine is large. As the work progressed, it became apparent that the hourly uric acid output during brief fasts is influenced by different factors, which for the most part we have been unable to account for satisfactorily. In order to find whether other urinary constituents were subject to parallel fluctuation with the uric acid, the work was extended to include creatinine and total nitrogen. An examination of the literature has failed to bring to light satisfactory data regarding this point.

The subject of the experiments is 22 years of age and weighs 55 kilos. His activity during the experiments consisted of laboratory routine. The analytical methods employed were the following: total nitrogen, Kjeldahl; creatinine, Folin's colorimetric

[1] Mendel, L. B., and Stehle, R. L., *J. Biol. Chem.*, 1915, xxii, 215.
[2] Folin, O., and Shaffer, P. A., *Z. physiol. Chem.*, 1901, xxxii, 552.
[3] Benedict, S. R., *The Harvey Lectures*, 1915–16, xi, 346.

method; uric acid, the Folin-Denis method as modified by Benedict and Hitchcock.[4]

The routine of the experiments was as follows. For 3 days before each experiment the diet was essentially free from purines. No food was eaten from 6 p.m. on the day preceding an experiment until after the experiment was finished. The urine was collected in hourly periods from 7 a.m. to 9 p.m., special care being taken to empty the bladder completely each time. The urines collected up to 4 p.m. were analyzed the same day, the later samples were preserved with toluene and analyzed the day following.

Table I, Experiments 1 and 2, records experiments in which the uric acid output only was determined and in which the quantity of water ingested was low but was not measured. The results corroborate the previous findings of Smetánka[5] and of Mendel and Stehle. There is a marked fall in the uric acid output during the morning hours, which is followed by a more gradual decline during the afternoon. An inspection of the tables will show, in agreement with the work of Mendel and Stehle, that there seems to be an unmistakable relationship between urinary volume and uric acid excretion, but only within certain limits. As a rule a decrease in urinary volume is accompanied by a decrease in uric acid, but numerous exceptions to this may be noted in the experiments, particularly in the early morning hours.

Experiment 3, Table I, records an experiment in which neither food nor water was ingested. The urine was collected in periods of approximately 3 hours. This experiment differed from the others reported in this paper in that a purine-containing food (chicken) was ingested about 5 o'clock in the afternoon preceding the experiment. The results illustrate the relation of uric acid to urinary volume, but do not appear to differ materially from those obtained in the other experiments.

[4] Benedict, S. R., and Hitchcock, E. H., *J. Biol. Chem.*, 1915, xx, 619. Dr. Benedict has modified the preparation of the Folin-Denis uric acid reagent as follows: Boil together 100 gm. of sodium tungstate, 30 cc. of 85 per cent phosphoric acid, 20 cc. of concentrated hydrochloric acid, and 750 cc. of water under a reflux condenser for 1½ hours. This modified solution shows less tendency to develop turbidity than does the original Folin-Denis reagent. Cool and make up to 1 liter.

[5] Smetánka, F., *Arch. ges. Physiol.*, 1911, cxxxviii, 217.

In Table II are recorded four experiments in which the intake of water was constant (100 cc. per hour), and in three of which the total nitrogen and creatinine have been determined for each hour. With a constant intake of a fairly liberal quantity of water the excretion of uric acid is somewhat more regular than in our first experiments (where the water intake was low but was not measured) or as compared with Experiments 8 and 9, where the water intake was low or irregular. The figures are of value as showing the variations to be expected in the same individual under apparently identical conditions. Particular attention is drawn to Experiment 5 which serves to illustrate a serious source of error in interpreting results upon the uric acid output. If, in Experiment 5, some material had been ingested at about 1 or 2 o'clock the inference might have been drawn that it increased the uric acid elimination, whereas the experiment really shows only the variation to be expected without ingestion of anything but a constant quantity of water throughout. It is probably safer, in interpreting uric acid results, to take as a basis a series of experiments such as is here given, rather than to rely upon the "control" period covering a few hours before the ingestion of the material to be studied.

A comparison of the figures for uric acid with the total nitrogen in Experiments 5, 6, and 7 will show that there is a remarkably close correspondence in the rise and fall of these two constituents. A given quantity of nitrogen in gm. does not correspond to a given weight of uric acid in the different experiments, but the relative rise and fall of the two are so nearly constant as to lead one to suspect that either may be regarded as a general index of kidney efficiency with respect to both. This point is one which we believe should be taken into account in any study of the effects of various agents upon uric acid formation. From our results it appears not improbable that when the kidney is called upon to do additional work in the excretion of one constituent it may respond by increased efficiency in respect to other constituents.

The relation between uric acid elimination and the creatinine output is not quite so definite as that between uric acid and the total nitrogen. In a general way the creatinine elimination also parallels the total nitrogen, but it will be seen that this relation is not so constant as for the uric acid.

In connection with the data on the creatinine elimination, it is of interest to note that the hourly output of this constituent during brief fasts is apparently not nearly so constant as Shaffer[6] and Van Hoogenhuyze and Verploegh[7] have reported for individuals on normal diets. The fluctuations are large. In Experiment 5, for instance, there is an increase from 53 to 81 mg. between two successive hours, an increase of over 50 per cent. Special care was used in such instances to check up the analytical work, determinations being made in triplicate wherever marked variations were found between two successive hours. The marked creatinine fluctuations recorded in Experiments 5, 6, and 7 are obviously not due to incomplete emptying of the bladder. In Experiment 8 (Table III), where 47.1 mg. of creatinine are eliminated for the period from 6 to 7 followed by 60.4 g. from 7 to 8, it might be held that the bladder was not completely emptied at 7 o'clock. Reference to the total nitrogen will show, however, that this constituent practically doubled for the hour 7 to 8 as compared with 6 to 7, and as there is no relation between this increase and that of the creatinine and uric acid on a percentage basis, it is obvious that an explanation based upon incomplete emptying of the bladder is not tenable.

We believe that the experiments which are here reported demonstrate beyond doubt that for short fasts the creatinine output may show marked variations from hour to hour. In nearly all of our experiments there is a tendency for a very low creatinine elimination during 1 hour of the late afternoon or early evening. This point is also shown in the experiments recorded in Table III, in which the water intake is markedly different from the earlier ones. The uric acid output in these latter experiments is hardly as regular or constant as in Experiments 4 to 7. The effect of varying the water intake is not, however, clear-cut.

In conclusion, I wish to express my thanks to Dr. S. R. Benedict for his helpful suggestions throughout this work.

[6] Shaffer, P., *Am. J. Physiol.*, 1908–09, xxiii, 1.

[7] Van Hoogenhuyze, C. J. C., and Verploegh, H., *Z. physiol. Chem.*, 1905, xlvi, 415.

Experiment 1. August 8. Fasting. Water Taken Every Hour. Quantity Not Measured.

Hour.	Volume.	Uric acid.
	cc.	mg.
7– 8	44	25.0
8– 9	25	18.7
9–10	27	21.5
10–11	19	14.4
11–12	14	11.7
12– 1	17	13.9
1– 2	18	11.5
2– 3	15	11.6
3– 4	12	9.7
4– 5	16	9.5
5– 6	12	9.6
6– 7	14	9.5
7– 8	22	7.1
8– 9	14	4.7
Total..................	269	178.4

Experiment 2. October 3. Fasting. Water Taken Every Hour. Quantity Not Measured.

Hour.	Volume.	Uric acid.
	cc.	mg.
7– 8	39	21.4
8– 9	50	11.5
9–10	98	23.0
10–11	69	18.7
11–12	71	15.6
12– 1	14	6.8
1– 2	41	10.0
2– 3	52	11.8
3– 4	12	5.8
4– 5	22	6.8
5– 6 } 6– 7 }	45	15.0
7– 8	70	9.9
8– 9	176	12.6
Total..................	759	168.9

Experiment 3. October 7. Fasting. No Water.

Hour.	Volume.	Hourly volume (average).	Uric acid.	Hourly uric acid (calculated).
	cc.	cc.	mg.	mg.
7–10	195	65.0	67.4	22.4
10– 1	61	20.3	42.8	14.2
1– 3.45	39	14.0	23.8	8.6
3.45– 6	36	16.0	27.2	12.0
Total.........	331		161.2	

TABLE II.

Experiment 4. October 24. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Total nitrogen.
	cc.	*mg.*	*gm.*
7– 8	64	20.6	
8– 9	93	26.8	
9–10	118	28.8	
10–11	122	18.7	
11–12	92	16.2	
12– 1	60	14.0	
1– 2	58	13.9	
2– 3	27	13.0	
3– 4	26	10.6	
4– 5	24	11.7	0.313
5– 6	14	7.1	0.185
6– 7	30	10.7	0.428
7– 8	23	9.9	0.324
8– 9	66	9.9	0.451
Total............	817	211.9	

Experiment 5. November 2. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	56	25.2	60.6	0.362
8– 9	148	24.0	60.7	0.441
9–10	62	21.8	60.0	0.415
10–11	38	19.0	49.8	0.300
11–12	37	15.4	49.8	0.302
12– 1	25	14.2	51.0	0.272
1– 2	36	15.4	49.8	0.364
2– 3	47	16.0	50.6	0.382
3– 4	54	19.0	64.0	0.418
4– 5	53	16.0	53.4	0.331
5– 6	58	23.4	81.0	0.517
6– 7	97	16.6	64.8	0.482
7– 8	49	16.8	53.4	0.343
8– 9	57	16.6	53.4	0.361
Total.........	817	259.4	802.3	5.290

TABLE II—*Concluded.*

Experiment 6. November 9. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	42	25.3	52.0	0.394
8– 9	64	25.4	54.5	0.471
9–10	73	25.2	56.0	0.517
10–11	50	22.5	50.5	0.405
11–12	95	18.2	54.0	0.466
12– 1	53	17.2	52.8	0.393
1– 2	43	16.8	52.0	0.395
2– 3	35	16.4	50.5	0.376
3– 4	26	16.4	51.2	0.332
4– 5	35	17.0	53.1	0.404
5– 6	37	13.6	50.5	0.389
6– 7	25	10.5	37.6	0.276
7– 8	39	13.1	53.8	0.412
8– 9	68	17.5	59.1	0.556
Total.........	685	255.1	727.6	5.786

Experiment 7. November 16. Fasting. Water, 100 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	41	20.8	40.5	0.362
8– 9	41	20.8	50.5	0.440
9–10	50	27.8	54.0	0.504
10–11	32	16.8	41.6	0.377
11–12	62	17.0	48.0	0.482
12– 1	56	16.0	49.2	0.468
1– 2	34	14.8	48.4	0.376
2– 3	20	13.2	48.0	0.340
3– 4	23	12.7	48.2	0.383
4– 5	33	11.2	48.4	0.402
5– 6	41	10.6	51.2	0.476
6– 7	154	11.4	58.9	0.560
7– 8	31	10.8	42.6	0.315
8– 9	38	12.5	46.0	0.452
Total.........	656	216.4	675.5	5.937

TABLE III.

Experiment 8. December 5. Fasting.

Water: 100 cc. hourly to 2 p.m.
200 " at 2 p.m.
150 " hourly to 7 p.m.
250 " at 7 p.m.
200 " " 8 "

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen
	cc.	*mg.*	*mg.*	*gm.*
7– 8	67	24.1	57.3	0.420
8– 9	77	25.6	62.3	0.454
9–10	171	22.5	61.2	0.539
10–11	132	17.6	56.7	0.439
11–12	34	15.4	49.8	0.293
12– 1	37	16.0	56.8	0.358
1– 2	41	16.0	60.5	0.397
2– 3	66	13.5	54.0	0.375
3– 4	69	12.0	57.8	0.451
4– 5	93	9.0	52.0	0.426
5– 6	25	Lost.		
6– 7	26	8.6	47.1	0.256
7– 8	164	11.1	60.4	0.478
8– 9	275	9.8	57.8	0.533

Experiment 9. January 4. Fasting. Water, 50 Cc. Each Hour.

Hour.	Volume.	Uric acid.	Creatinine.	Total nitrogen.
	cc.	*mg.*	*mg.*	*gm.*
7– 8	73	25.0	60.6	0.505
8– 9	83	25.5	62.6	0.516
9–10	77	23.0	62.9	0.484
10–11	43	21.4	59.6	0.351
11–12	30	22.0	58.9	0.347
12– 1	22	18.8	59.2	0.312
1– 2	27	17.9	60.8	0.340
2– 3	16	12.5	50.0	0.230
3– 4	17	12.5	60.3	0.265
4– 5	14	9.6	55.5	0.237
5– 6	13	9.1	46.0	0.215
6– 7	22	17.3	65.6	0.332
7– 8	23	11.8	51.3	0.311
8– 9	19	9.8	41.6	0.273
Total.........	479	236.2	794.9	4.718

CORN AS A SOURCE OF PROTEIN AND ASH FOR GROWING ANIMALS.

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Agriculturists have long since recognized the inadequacy of the corn kernel as the sole diet of growing animals, and investigators in the field of nutrition have made numerous studies to determine more exactly the nature of its deficiencies. An earlier publication of the author presented some recent data showing that the first limiting factor in corn for the growth of young rats is the lack of certain inorganic constituents. When the mineral deficiencies were corrected, however, normal growth could not be secured, even after the addition of considerable quantities of purified protein, indicating a lack of suitable growth accessories. McCollum and his collaborators have presented data bearing on this problem, and state that the maize kernel is lacking in one of the accessories, which they have designated as fat-soluble A.

Our earlier investigations disclosed some minor variations in the way swine and rats responded to an exclusive diet of maize. For example, the mineral deficiencies of corn were tolerated much better by the swine, while the protein deficiencies were tolerated much more easily by the rats. Assuming that corn is poor in one or more of the growth accessories, swine are affected much less injuriously by this than are the rats.

Our earlier work has been extended, and efforts are now being made to determine specifically what inorganic elements in the ash and what amino-acids in the proteins are deficient in quantity, and thus constitute limiting factors when the corn kernel is used as the exclusive diet of young animals. Data from this laboratory have been presented[1] showing that the addition of trypto-

[1] Hogan, A. G., *Proc. Am. Soc. Animal Production*, 1916 (in press).

phane and lysine improved the proteins of corn, but our results at that time did not indicate which of these amino-acids was the first limiting factor in the protein mixture of the corn kernel. Our later results have established that point.

The methods used in our earlier work were followed closely in these feeding trials, and in most instances rats were used as experimental animals. In some cases swine were used, but only to a limited extent.

I. The Inorganic Limitations of the Corn Kernel.

Because of the inadequacy of the proteins in corn they were supplemented by a comparatively ash-free protein, a specially prepared specimen of egg white. The analyses of corn make it probable that the animals receiving this grain as an exclusive ration will first require calcium to permit the normal nutritive processes, and this view seems upheld by the facts. One lot of animals received corn plus the ash-free protein, and other lots received this same ration with various mineral supplements. In one case the supplement was a complete ash mixture;[2] in another it was calcium lactate, and in another potassium phosphate. In the two latter cases the animals received the same quantities of calcium lactate and potassium phosphate as the first lot received in the complete ash mixture. During the period of observation the lot receiving the complete ash mixture grew somewhat more rapidly than that receiving corn plus the calcium lactate. The rats receiving corn plus potassium phosphate profited in no way by the addition, and died about as quickly on the ration as those receiving no mineral supplement whatever. The results appear in Table I. Rats were used as experimental animals.

	gm.
[2] Ca lactate	468.0
K_2HPO_4	280.8
NaCl	123.1
Na citrate	31.2
Fe citrate	23.8
$MgSO_4$	31.7

TABLE I.

Mineral Supplements for Corn.

Ration.......	Corn + ash-free egg white.		Corn + ash-free egg white + K_2HPO_4.		Corn + ash-free egg white + Ca lactate.		Corn + ash-free egg white + complete ash mixture.	
Sex and No....	♀ 1	♂ 1	♀ 3	♂ 3	♀ 2	♂ 2	♀ 2	♂ 1
Time.	Average weight, *gm.*							
wks.								
Initial.	50	52	46	49	50	49	66	56
3	64	78	50	48	71	82	95	85
6	50	72	60	57	75	98	102	107
9	Dead.	Dead.	Dead.	57	85	116	114	132
12				Dead.	89	124	124	177
15						132	125	180
18						146	130	175
21							135	215
24							135	225
27								225

II. The Amino-Acid Deficiencies of Corn Proteins.

Of the individual proteins of corn, zein has received the most attention from investigators. According to Osborne and Mendel (1914, *a*), it makes up about 40 per cent of the total nitrogenous constituents. It contains neither lysine nor tryptophane, and as has been known for some time, is unable to support life when it forms the only protein of the ration. Willcock and Hopkins used young mice in feeding trials, and obtained some of the earlier data bearing on this point. When receiving a diet containing no other protein than zein, their mice lived only a short time. When tryptophane was added to the ration, life was much prolonged. Some years later Osborne and Mendel (1914, *b*) showed that zein could support life and even permit growth if it were supplemented with lysine and tryptophane.

Although there is no question that these amino-acids must be added to zein in order that it may support growth in the animal organism, it is not so certain that the mixture of proteins in the entire corn kernel would show a corresponding deficiency. It has been demonstrated by Osborne and Mendel (1914, *a*) that corn glutelin is an adequate protein, and there is a general im-

pression that the proteins of the corn germ are relatively very effective. Some data in support of that view have been obtained in previous years at this Institution. One lot of swine received corn alone. Other lots received corn with the addition of various supplements, including casein and corn germ. Table II gives further details concerning the rations, as well as the growth of the animals.

TABLE II.

Corn Germ as a Source of Protein for Growing Swine.

Lot No.	36	29	34	23	31
Nutritive ratio	1:6	1:6.9	1:8.8	1:8.0	1:8.8
Ration	Corn + casein + salt mixture.	Corn + casein.	Corn + starch + casein + salt mixture.	Corn + corn germ.	Corn + salt mixture.
	per cent	*per cent*	*per cent*	*per cent*	*per cent*
Protein, corn	10.29	11.39	5.64	7.74	10.88
Protein, supplement	4.21	1.22	4.78	5.08	
Ether extract	4.97	4.19	3.02	7.20	4.58
Nitrogen-free extract	76.15	81.40	82.80	77.20	80.30
Ash	4.33	1.71	3.73	2.79	4.27
	lbs.	*lbs.*	*lbs.*	*lbs.*	*lbs.*
Average initial weight	21	31	24	31	25.3
Average final weight, (180 days)	200	142	165	150	37.6

Lots 29 and 23 received tap water, high in calcium. The others drank only distilled water. There were three animals in each lot.

The table presents in a striking way the vast superiority, for growth production, of casein over the corn proteins. For all practical purposes it may be said that Lots 29 and 31 received the same diet, with the exception that casein to the extent of only 1.22 per cent of the entire ration was added to the feed of Lot 29. Yet this comparatively small addition changed the ration from one that permitted practically no growth to one that permitted approximately two-thirds normal growth. Possibly a suitable mineral supplement would have augmented this difference, for obviously Lot 29 was receiving no excess of mineral matter. A comparison of Lots 31 and 34 shows the superiority

of the casein in an equally convincing manner. Each lot received the same amount of digestible protein, assuming that the casein was entirely digestible. The fact that half this protein was casein, however, changed the ration from one that would barely sustain life to one that permitted a degree of growth not far from normal. Brief consideration of Lots 23 and 34 is also of interest. A strict comparison is impossible as the composition of the rations is quite dissimilar. Obviously, however, the proteins in the corn germ are effective in supporting growth of young animals. It would not be impossible, therefore, that the proteins of corn germ contained high percentages of the amino-acids lacking in zein, and might at least in some measure compensate for the deficiencies of the latter protein.

In our earlier work lysine and tryptophane were added to finely ground corn, and this mixture was fed. This addition had no apparent value, but the results were not considered decisive because rats failed to grow notably better when our purified proteins, such as egg white, were added to corn. It seemed evident therefore that some other factor than the quality of protein should be considered. This may have been a suitable mineral supplement, growth accessories, or some less considered question. In order to obviate all such possibilities, it seemed desirable to use a ration known to be adequate in every respect other than protein. For that purpose a ration[3] approximating some published by Osborne and Mendel was adopted. At first the rats received approximately 9 per cent protein, but later this was increased to 12 per cent. Corn itself seldom contains much over 10 per cent protein, so in order to have 12 per cent in the ration after admixture with other constituents, it was necessary to obtain the protein in a more concentrated form. Corn gluten did not seem suitable, as it lacks the corn germ. Accordingly efforts were made to obtain a product in which the natural mixture of

[3] The ration was as follows.

	Protein.	
	9 per cent.	12 per cent.
	gm.	gm.
Corn protein	285.0	360.0
Protein-free milk	250.0	250.0
Butter	250.0	250.0
Starch	260.0	138.0

corn proteins was modified as little as possible, but which contained them in larger proportion. The corn was ground to an impalpable powder, and boiled in a large volume of water made slightly acid with acetic acid. The purpose was to coagulate the soluble proteins, and at the same time bring the starch into a colloidal solution. After standing some time the protein settled and then the supernatant liquid was siphoned off. This process was repeated five or six times, until the liquid did not give the iodine test for starch. The product obtained in this way contains 30 to 35 per cent protein, and is suitable for use in the feeding mixture mentioned above.

At the beginning of the feeding period, one lot of rats received the mixture containing the prepared corn proteins, and another lot of six received the same ration with the addition of lysine and tryptophane. The amount of corn protein in this basal ration was purposely made very low, in order that growth should occur very slowly. Under these circumstances, any increase in the rate of growth due to the added amino-acids should be clearly perceptible. The amounts of these supplements were relatively small; to each 1,000 gm. of the feed, 3 gm. of tryptophane and 3 gm. of lysine dichloride were added. Two of the rats receiving the mixture containing no added amino-acids died relatively soon, and another died near the close of the experiment. These are not included in the charts. None of the rats receiving the additional amino-acids died, and though they did not grow rapidly, yet in the first 16 weeks they made over three and a half times the gain that the other lot did. At the end of 17 weeks, the lysine and tryptophane were removed from the diet, and these rats promptly began losing weight. After a short interval, tryptophane was added to the diet of three of these animals, and both lysine and tryptophane to the ration of the other three. All began growing again, but those receiving lysine and tryptophane grew much more rapidly than those receiving only tryptophane. Later the animals were given the basal ration with lysine alone added, and without exception they lost weight at once, showing clearly that tryptophane is the first limiting factor in the corn proteins. After these proteins are supplemented with tryptophane, the addition of lysine again accelerates the rate of growth, and the latter amino-acid is evidently the second limiting factor. More complete data are presented in Charts 1, 2, and 3.

EXPLANATION OF CHARTS.

All animals received the same basal ration at all times. At the point indicated by *a*, the protein was increased from 9 per cent of the ration to 12 per cent; the point *b* indicates a further increase to 15 per cent protein. In addition to the basal ration, two of the animals shown in each chart received at various times lysine and tryptophane, given together and separately. The numbers in the upper left hand corner signify which animals received the amino-acids, and the heavy lines accompanying them indicate the time during which these supplements were administered. A gap in a line shows that none of that particular amino-acid was received during the interval thus indicated. It is evident from inspection of these charts that the addition of lysine alone did not cause the rats to grow more rapidly. The animals did grow more rapidly, however, when tryptophane alone was added, and still more rapidly when both amino-acids were added.

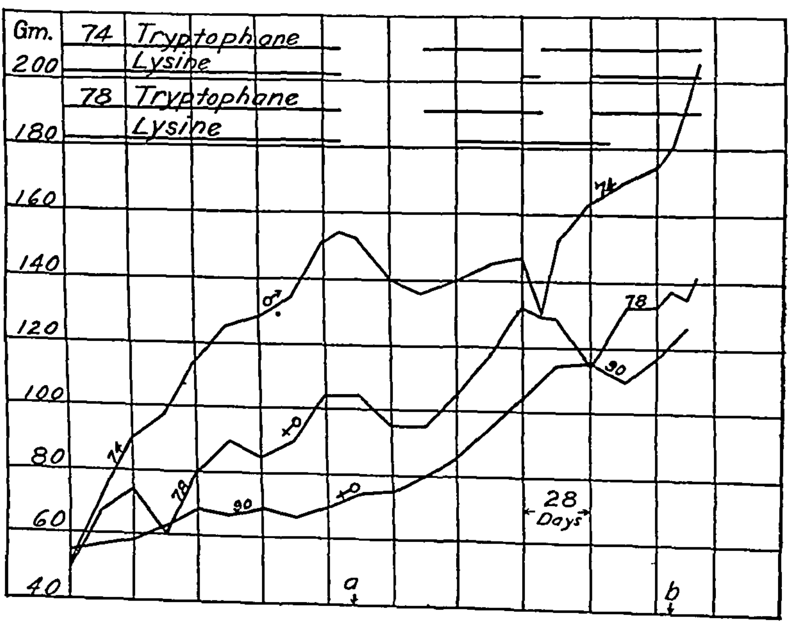

CHART 1.

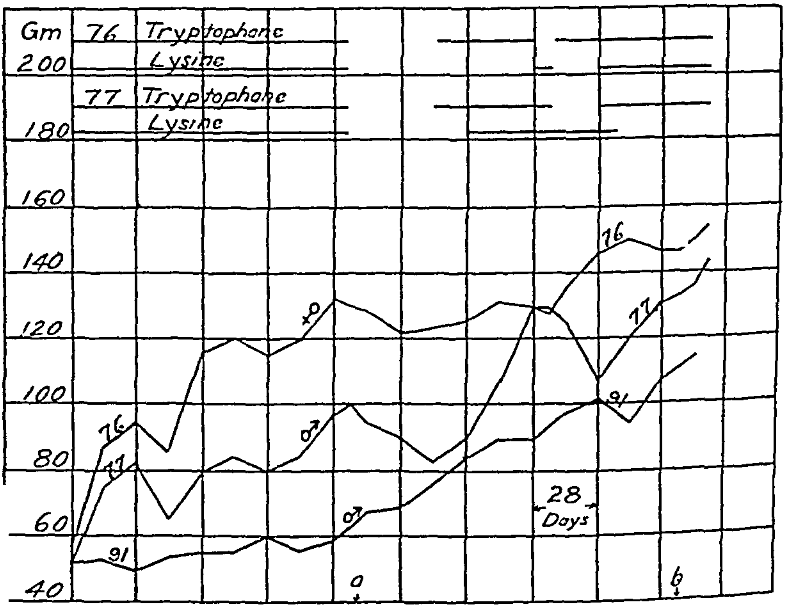

CHART 2.

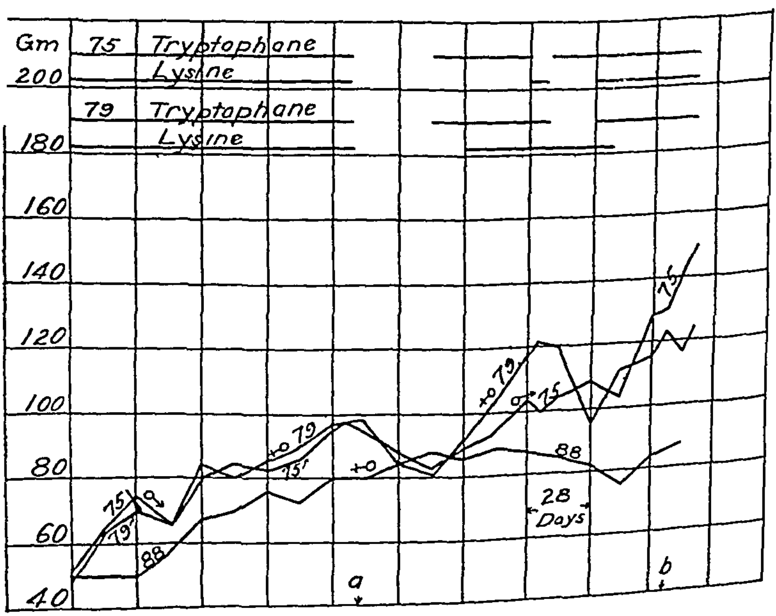

CHART 3.

SUMMARY.

The most important mineral deficiency of corn is calcium. Tryptophane is the first limiting factor in the proteins of the corn kernel, and then lysine.

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THE CHEMICAL NATURE OF THE "VITAMINES."

III. THE STRUCTURE OF THE CURATIVE MODIFICATIONS OF THE HYDROXYPYRIDINES.

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PLATE 4.

(Received for publication, February 28, 1917.)

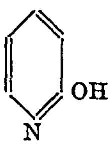
In the first paper of this series (1) it was shown that α-hydroxypyridine exists in two crystalline forms differing in physiological properties. These were assumed to be distinct isomeric substances. Moreover, evidence was found that the sodium salt of the substance represents a third (enol) form. One of the modifications was obtained as needles and possessed remarkable curative power for polyneuritis in pigeons, but under ordinary conditions spontaneously changed into crystalline granules which were physiologically inactive. Such a change of physiological properties appears to take place under similar conditions in all the mono- and polyhydroxypyridines so far studied.

In order to be of value in elucidating the chemical nature of the "vitamines" occurring in foodstuffs, the discovery of this physiologically important transformation must be supplemented by definite evidence of the chemical structure of the curative form. It will further be necessary to distinguish sharply between those structural features which are indispensable to the antineuritic property and those which play a secondary, insignificant, or adverse rôle. Some initial steps in this direction constitute the main subject of the present paper.

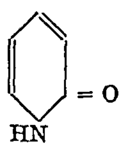
Structure of the Curative Substance.—The difference in physiological activity in the two crystal forms is sufficient to prove that they are distinct substances. Analyses and molecular weight determinations confirm the earlier assumption that they are iso-

meric. All the evidence indicates that the stability of each form is determined by temperature, solvent, etc., and that the two modifications are readily interconvertible by proper adjustment of conditions. These facts suggested a labile hydrogen atom.

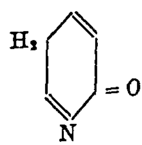
That there should exist some form of lability of the hydrogen atom of the hydroxyl group of α-hydroxypyridine seems wholly probable if one may reason by analogy to phenol. In fact a lability of this atom appears to have been proven by the production of two isomeric alkyl derivatives, in one of which the alkyl radical is attached to oxygen and in the other to nitrogen (2). On the other hand, there is no reason to suppose that any other atom in the molecule shows a similar lability. Therefore it is assumed *that all the isomeric rearrangements of α-hydroxypyridine involve the shifting of only one atom; i.e., the hydrogen of the putative hydroxyl group.* If the truth of this assumption is granted, the compound theoretically may exist in six isomeric forms as follows:



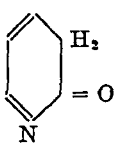
I. Enol.



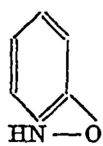
II. Pyridone.



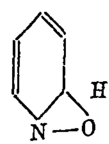
III. Para-quinonoid



IV. Ortho-quinonoid.



V. Pseudo betaine.



VI. Anthranil.

Two of these formulas represent the hydrogen as attached to nitrogen, three as attached to carbon, and one as attached to oxygen.

By a study of the methyl ethers of α-hydroxypyridine it was possible tentatively to eliminate Formulas I, III, IV, and VI as representative of the curative form, for it was found that while methoxypyridine is non-curative and toxic, the nitrogen methyl derivative shows distinct indications of curative properties.

It has been definitely proven that in the so called methylpyridone, the methyl group is actually attached to nitrogen

since methylamine is produced by reduction with sodium amalgam (2). But formulas corresponding to both II and V have been assigned to this compound (3) and the adherents to the respective theories have not recorded any agreement on the question. Kauffmann regarded the structure shown in Formula V as definitely proven by the fact that the vapors of the substance, under the influence of the Tesla current, glow with a blue luminescence similar to that produced by pyridine and benzene under the same conditions (4). He looked upon this as conclusive evidence of the presence of three pairs of shifting double bonds. Actually this evidence does not exclude the possibility of the existence of two forms in equilibrium. We have equally definite evidence that the substance does exist in a second form in the fact that it is strongly basic and forms well defined salts with acids. Such properties are impossible in a compound in which the only nitrogen atom is already pentavalent. Further reference will be made to this matter later.

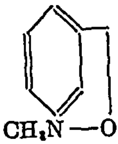
In view of the existing uncertainty regarding the structure of methylpyridone it was impossible to decide between Formulas II and V for the curative form of α-hydroxypyridine, even if the tentative exclusion of the remaining formulas should prove valid. Formula II has been generally accepted for the needle (curative) form, which is the only crystallographic modification heretofore observed. But the literature (5) does not describe a single experimental fact which cannot be interpreted according to either formula, and no additional chemical or physical evidence bearing on the subject has been found as yet. However, the physiological properties of β-hydroxypyridine (6) render a conclusion possible regarding the structure of the curative form of the free and substituted hydroxypyridines, regardless of the existence or structure of other forms. A structure corresponding to Formula II is obviously impossible for β-hydroxypyridine, as has been pointed out by Pechmann and Baltzer (2), since the valences of all the atoms of the ring cannot be satisfied on this assumption. The fact that β-hydroxypyridine under proper conditions shows physical, chemical, and curative properties similar to those of the α derivative therefore enables us to exclude Formula II and come to a decision that the curative forms of all the hydroxypyridin possess a structure best represented by formulas of type V

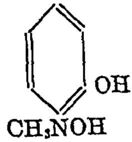
conclusion which is confirmed by further evidence presented later in this paper.[1]

If β-hydroxypyridine exists in two forms and the structure of the curative modification is of type V it is safe to predict the existence of a second methyl derivative VII (7). A further study of the chemical and physiological properties of β-hydroxypyridine is in progress and all experimental data regarding it will be reserved for later publication.

Structure of Non-Curative Modifications.—Pending further work the structure of the non-curative forms of the hydroxypyridines and the alkyl pyridones remains in doubt. We may reasonably conclude that the crystalline non-curative forms of monohydroxypyridines are not enols since they do not instantaneously absorb bromine in cold alcoholic solution. Whether they are derivatives of the pyridone (II) or of the para- (III) or of the ortho- (IV) quinonoid type it is impossible to say, though the predominance of para-quinonoids in the benzene series would argue in favor of Formula III. Formula VI seems altogether improbable. In view of the experimental evidence described in the literature, supplemented by physiological tests referred to in the experimental part of this paper, it seems probable that methylpyridone exists in a betaine-like form (V) and also in the form of a hydrate (VIII) or possibly an isomer (IX) or both. The difficulty with which the substance is freed from water suggests the hydrate (8).

[1] It will be understood that the possible imperfections of the conventional methods of representing chemical structure are not taken into account in this conclusion. There is much to suggest that the dimorphism of α-hydroxypyridine may be akin to what has been loosely called "physical isomerism." In the writer's opinion this conception is in itself a contradiction in terms. If two solid modifications of any substance such as tin or phosphorus possess different proportions of free or available energy, they must differ from one another structurally. In the absence of evidence to the contrary the free chemical energy must be presumed to arise from the same source as do those forces which we speak of as valences. That is to say, the free energy must be in the molecule, not deposited upon it.


VII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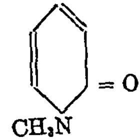

IX

Betaine-Like Character of the Curative Form.—If the structure of the curative form of α-hydroxypyridine is indicated in Formula V it is essentially a betaine. Experimental results indicate that there is an analogy between the two crystalline forms of α-hydroxypyridine on the one hand and the α-amino-acid esters and their corresponding betaines on the other in respect to their mutual convertibility at various temperatures. Willstätter (9) has found in the case of the methyl ester of dimethylamino-acetic acid and the isomeric betaine that both forms are stable below 135°; between 135° and 293° the betaine form exists to the exclusion of the ester, and above 293° the ester becomes more stable than the betaine. A similar relation according to Willstätter exists in all the α-betaines. In α-hydroxypyridine the interconversion of the two forms apparently takes place with greater ease than the rearrangements studied by Willstätter but are probably of a similar nature, as the former involves the shift of a relatively labile hydrogen atom as compared with the less mobile alkyl groups with which Willstätter was concerned. The conversion of methylpyridones into corresponding methoxypyridines by heat (10) also presents a close analogy to α-betaines in which the methyl group may similarly migrate from nitrogen to oxygen. The hypothetical formation of a hydrate of methylpyridone in water solution also conforms to the chemistry of betaines.

In view of this similarity it seemed wise to attempt a physiological study of some compounds, the betaine structure of which is generally accepted. Some years ago the author made a few tests of the antineuritic properties of trigonelline (hydrate) which resulted entirely negatively (11). Drummond and Funk (12) also reported that betaine (trimethyl glycine) salts are devoid of curative action. But on closer inspection it will be seen that these experiments are not conclusive evidence on the point in question, since in the classes of betaines as a whole the characteristic be-

taine ring is not known to persist in water solution nor in the hydrated crystalline forms, and certainly does not exist in the salts of these bases (13). Betaines are essentially anhydrides which take up water (or acids) with greater or less ease. In view of this fact it seemed unlikely that any antineuritic properties which the commoner betaines might possess in the anhydride form could actually be demonstrated, since physiological processes take place in aqueous media. Notwithstanding this obvious difficulty a few birds were treated by the expeditious injection of freshly made cold water solutions of dehydrated trigonelline and betaine. The results obtained with trigonelline under these conditions were inconclusive. With betaine it was found possible to effect substantial improvement or practically complete cures of the paralytic symptoms in nearly all cases, so that no doubt can exist that the substance is curative to a degree at least as long as the anhydride form persists.

The Feature Essential to Antineuritic Properties.—The curative action of betaine is important, first, in confirming the structure of the curative form of the hydroxypyridines and, second, in showing that a labile hydrogen atom is not essential to such properties. That a subordinate or negligible rôle is played by the labile hydrogen atom is further indicated by the results obtained with methylpyridone and trigonelline. The distinct beneficial effects upon polyneuritis of betaine as well as of allantoin and certain purines and pyrimidines (14), and the evidence cited in the second paper of this series bearing on the antineuritic properties of adenine (15), indicate that the pyridine ring is not requisite to some such physiological properties. Judging from curative tests alone one might infer that a betaine-like structure is an essential and highly important feature of natural "vitamines." However, attempts to protect birds against polyneuritis by the regular administration of methylpyridone, trigonelline, or betaine have failed. This suggests that the response of polyneuritic birds to treatment with such substances is a phenomenon not intimately connected with true antineuritic action. But such a view seems less reasonable than that the failure of the synthetic substances in protective experiments is due to the extreme instability of their active forms, their toxicity, or other properties which render them incompletely adaptable to the uses of the

animal organism. Accordingly, it is assumed as a working hypothesis that such a betaine-like structure constitutes an essential part of "vitamine" molecules.

Distribution of Betaine-Like Ring in Nature.—If this reasoning proves to be correct, it will still fail to assist greatly in establishing the identity of the natural "vitamines," for a betaine-like structure is theoretically possible in a large proportion of the known nitrogenous constituents of animal tissue. It may exist in any substance containing a basic nitrogen atom in the proximity of a labile hydrogen atom of an alcohol, phenol, carboxyl, or amino group. Aside from the amino-acids and their alkyl substitution products, for many of which both betaine and straight chain modifications have been proven, there is another much larger class of compounds in which a betaine structure is possible. It includes all the amino and oxy derivatives of cyclic nitrogen compounds, of which the oxypyrimidines may be regarded as representative. In some of these substances a shifting of hydrogen from oxygen to nitrogen has been proven by the formation of both oxygen and nitrogen ethers. This form of tautomerism commonly has been expressed as follows:

However, all the facts would be accounted for equally well by the assumption of the existence of the following modifications:

In most instances no purely chemical means would be adequate to determine which conception is correct and neither can be dismissed as improbable. Modifications of either or both types may exist. The work of Hans Meyer (16), of Geake and Nierenstein (17), and of Griess (18), and the polymorphism of pseudo conhydrine (19) and 4-phenyl-isocytosine (20) are of interest in this connection. To determine definitely in what compounds and under what conditions betaine modifications actually exist may require many years of work. To decide which of such

compounds are sufficiently stable to be absorbed unchanged from the alimentary tract may prove another equally difficult problem. Progress in both cases would be greatly facilitated by the discovery of some physical means of detecting the betaine linking.

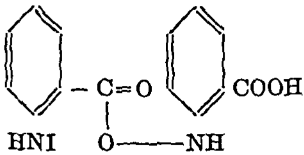
Nicotinic Acid.—While it is probable that most potential betaines do not function as "vitamines" in our foodstuffs, the assumption that the "vitamines" invariably belong to a narrowly restricted group of compounds is without adequate foundation. Evidence has been found suggesting that an isomeric form of adenine is responsible for antineuritic properties in a certain fraction of autolyzed yeast (15). Should this prove to be correct the writer still would not presume that such a form of adenine is necessarily the only antineuritic substance in yeast, much less in all nature.

Vedder and Williams (21) fractioned the filtrate from the decomposed phosphotungstates of hydrolyzed extract of rice polishings by precipitating with silver nitrate, first in neutral solution (purine fraction) and second in alkaline solution (Funk's "vitamine" fraction). The filtrate from the second precipitate constituted a third fraction. All these fractions protected fowls from polyneuritis, although only the second, or "vitamine" fraction, produced prompt cures of the disease. They recorded their conclusions as follows: "Therefore it appears certain that there are several groups of chemical substances that are capable of protecting fowls against polyneuritis gallinarum."

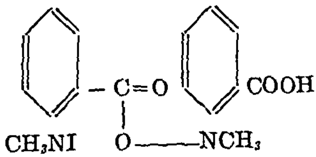
This is of particular interest in view of the obvious discrepancy in elementary composition between adenine and the substances obtained by Funk from his "vitamine" fraction of yeast and rice polishings. Adenine contains more than 50 per cent of nitrogen while the relatively pure crystalline substances from Funk's fraction, among which was nicotinic acid, contained only 10 to 12 per cent of this element. The possibility that the curative substance in Funk's "vitamine" fraction was actually a derivative of a tautomeric form of adenine seems very remote. In view of these considerations one can hardly escape the feeling that the presence of nicotinic acid in considerable proportions in Funk's "vitamine" fraction of both yeast and rice polishings is not accidental, and that Funk's conjecture, "It is not unlikely

that nicotinic acid is a decomposition product of the vitamine" (22) had some foundation in fact.

Turnau (23) has described a series of basic salts of the α- and γ-pyridine carboxylic acids and their methyl betaines. Thus a water solution of picolinic acid, to which is added an excess of hydriodic acid, on evaporation deposits a well defined basic hydriodide to which Turnau assigned the formula X. That is, he regards it as the hydriodide of picolinic acid picolinate. The corresponding methyl derivative XI possesses an obvious resemblance to a betaine.

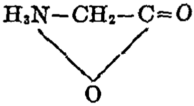


X

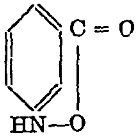


XI

Turnau did not succeed in preparing such derivatives of nicotinic acid but his work suggested the possibility of the existence of an isomer, polymer, or simple derivative of nicotinic acid in which the carboxyl group exists as a betaine ring. Whether or not Turnau's formulas are correct, if the aliphatic α-amino-acids under any conditions have the structure XII, there is no theoretical obstacle to the existence of nicotinic acid in the form represented by XIII, either free or in some simple combination, especially since both the methyl betaine and methyl ester of nicotinic acid are known.



XII



XIII

Accordingly, endeavors were made to convert nicotinic acid into an antineuritic form. On decomposing nicotinic acid hydriodide with silver oxide (24), the filtrate from the silver halide was found to possess marked curative properties. These results are such as to encourage further investigation with a view to the

preparation and isolation of such betaine derivatives, and finally to proving or disproving the "vitamine" character of nicotinic acid as it exists in certain antineuritic foodstuffs.

EXPERIMENTAL PART.

Pigeons were used as experimental animals, polyneuritis being developed by feeding exclusively on white rice. For the curative experiments described below birds were selected which had developed characteristic symptoms of the disease and showed these symptoms continuously in a severe form for several hours before treatment. This was done since it has been found that polyneuritic fowls sometimes suffer from mild, brief, or intermittent attacks of paralysis and subsequently recover without treatment, eventually developing chronic symptoms accompanied by emaciation and general weakness. Birds exhibiting the chronic form of the disease whether preceded by brief acute attacks or developing progressively were also excluded, since such birds cannot be treated with uniform success by any measures. Under this plan, of the total number of pigeons fed on white rice, only about 50 per cent were available for use, the losses including those birds which developed acute symptoms and died suddenly and those which failed to show severe, persistent paralysis within a reasonable time, usually about 35 days. The birds varied greatly in the incidence of the more characteristic type of the disease, a fact which has been insufficiently emphasized in recent literature. The writer has been unable to form a definite opinion as to the correlation of this incidence with the age, sex, appetite, or habits of pigeons, or with the precise degree of decortication of the rice used for feeding, or the season of the year chosen for experimentation. Any advantage which may be gained by forced feeding appears to be more than offset by the complications resulting from stuffed crops and excessive rough handling.

α-Hydroxypyridine.—Some further observations (1) have been made upon the conditions under which the two crystalline forms of α-hydroxypyridine may be transformed into one another. If the needle form is desired, it has been found advisable to heat the pure 6-hydroxynicotinic acid used for its preparation in an

oven at a temperature of 140° for several hours. The acid is then distilled in a flask previously thoroughly dried by baking and collected in a dry receiver protected from the moisture of the atmosphere by a calcium chloride tube. Under these conditions the colorless oily distillate remains in a clear fluid state for some minutes, especially if its volume is large. Crystallization gradually takes place largely in the form of needles often 2 or 3 cm. in length. Some granular crystals have always been obtained even under these conditions, especially in the first few drops of distillate. That part of the mass crystallizing in granules is opaque while the portions in which needles predominate remain semi-transparent sometimes for days and detached fragments have a sticky character. During the course of a few days the amount of granular crystals grows from the points of original contamination as evidenced by the spread of opacity and the decrease in the stickiness of detached crystals.

Masses of either modification if redistilled (boiling point 280–282°) do not undergo change of crystal form to any great extent. This would seem to indicate relative stability of both forms at that temperature. On the other hand, if the granular form is fused and the superfused mass is kept at 70–90° till crystallization is complete, the needle form is obtained exclusively. But if by sudden cooling to lower temperatures the deposition of the granular form once begins in such a superfused mass the whole mass will granulate rapidly and any needle crystals which have previously grown in the magma will be converted almost instantaneously into granular aggregates of needle shape, indistinguishable to the naked eye from true needle crystals. These facts indicate that at a temperature of about 70–90° the needle form is the more stable. On the other hand, at room temperature conditions are reversed and the granular form is stable, as will be seen below.

In solution the relative stability of the two forms has proven a baffling problem. From solutions of either form in ethyl or amyl alcohol, chloroform, or ether it has been found possible to obtain only the granular form. Crystallization always takes place slowly from such solvents so that presumably there is opportunity for rearrangement during the process.

If either form is dissolved in boiling benzene, and ligroin is

added to the cooled solution a milkiness is immediately apparent (25). If crystallization can be induced within a few moments needles will predominate, occasionally to the exclusion of the granular crystals. For some unknown reason the needle crystals can be more readily obtained in considerable proportions when the operation of recrystallization is carried out upon several grams of substance at once. If crystallization takes place slowly only the granular type is obtained. However, commonly, even with the greatest care, it is impossible to avoid the simultaneous formation of both types. When this occurs the needles first formed are rapidly converted into the granular condition if allowed to remain in the benzene-ligroin mixture and especially if stirred. Microscopic fields of the needle type slightly contaminated with granular crystals have frequently been seen to undergo complete transformation within a few minutes (Fig. 1). Dr. F. E. Wright of the Geophysical Laboratory kindly examined the two forms of crystals and watched the progress of this transformation. He summarized his observations as follows:

Stable Form.—This occurs either in irregularly shaped grains or in branching aggregates of short crystals with pyramidal terminations and so grouped that the crystals intersect at right angles. Under the microscope each crystal is seen to be twinned in such manner that the planes of optic axes of any two superimposed twinned portions intersect at right angles. No cleavage was observed. The crystals are colorless and strongly birefracting. The refractive indices are approximately: $\alpha = 1.61$, $\gamma = 1.78$. The optic axial angle 2E is large, at least 140°; optical character, negative; axial dispersion strong $2E_r > 2E_v$. On many of the sections the acute bisectrix and one optic axis appear in the field in convergent polarized light. So far as can be determined the interference figure shows a symmetrical distribution of the interference colors, as though the crystal system were monoclinic with inclined dispersion. Many of the crystals contain liquid inclusions with movable gas bubble.

Unstable Form.—This crystallizes in acicular lath-shaped crystals which, however, are not stable in the solution (*i.e.*, a benzene-ligroin mixture) but are rapidly dissolved and replaced by the stable form. The process of inversion appears to be largely one of actual solution with immediate precipitation, along the dissolving crystal, of grains of the stable form. The inversion does not appear to be a direct change in the crystal state from the unstable form to the stable form. The optical properties so far as determined are: optical character, negative; extinction parallel with positive elongation and with plane of optic axis parallel to elongation; birefringence medium to fairly strong, less than that in the stable phase.

If the crystals are quickly removed from the benzene-ligroin mother liquor and dried, the mixed forms persist for a longer time, but even in a solid state and in a dry atmosphere the conversion of needles into granular crystals continues to occur, though at a much reduced rate. The needles become merely roughly needle-shaped granular aggregates in the course of a few days or at most weeks. Intimate mixing of the two forms appears to hasten the change, which seems to proceed from points at which granular crystals exist. Accordingly, the rate of change varies over a wide range.

Water solutions of the needles remain physiologically active for at most only a few days when kept at ordinary temperatures. At boiling heat the curative property disappears promptly. When such solutions, whether old or freshly prepared, are evaporated, a residual syrup remains which solidifies to a crystalline mass. If crystallization takes place at ordinary temperature in a desiccator the crystalline substance is non-curative, but if maintained at 70–90° the syrup slowly deposits needles which are curative. The granular crystals may be dried at ordinary temperature over phosphorus pentoxide but do not thereby become curative. Both forms of crystals appear to melt at 106–107° and admixture of the two forms does not depress the melting point. Probably the two modifications do not actually possess the same melting point but no distinction can be made because the substances are interconvertible. The polymorphism of the compound has a striking resemblance to that of conhydrine and pseudo conhydrine (19), and transformations take place with such ease as to make impossible a chemical differentiation of the hypothetical isomeric modifications. Even the preparation and isolation of the metastable form require conditions beyond the complete control of the experimenter who must expect failures more frequently than successes. The tendency of the stable modification to form acicular aggregates is very deceptive. Observation under crossed Nicol prisms is useful for the detection of this phenomenon. Persistence of crystal form when moistened with a benzene-ligroin mixture may also be taken as satisfactory evidence of the presence of the stable phase.

An amount of the needle crystals was obtained in apparently a nearly pure condition by crystallization from benzene and li-

groin, as above described. The crystals were quickly collected on a suction filter, washed with petroleum ether, and dried for an hour *in vacuo* over sulfuric acid and paraffin. A weighed sample was used for the determination of the molecular weight by the depression of the freezing point of water.

0.5200 gm. substance in 9.6164 gm. of water depressed the freezing point 0.868°. Molecular weight found: 108.9. Calculated for C_5H_5NO: 95.05.

This determination is in substantial agreement with one made by Koenigs and Geigy (25) by the vapor density method. The crystal form of the substances as described by these authors was that of needles.

After completion of the cryoscopic determination a portion of the solution used for this purpose was administered to a polyneuritic pigeon by injection. A dose of 1 mg. of substance had completely relieved the paralysis by the following morning.

A sample of the granular form prepared by crystallization from benzene and ligroin as above described was washed with petroleum ether, pressed between filter papers, kept *in vacuo* over paraffin for 24 hours, and dried over phosphorus pentoxide for a week.

In a solution of 0.3787 gm. substance in 9.1535 gm. of water a depression of the freezing point of 0.744° was observed. Molecular weight found: 106.4. Calculated for C_5H_5NO: 95.05.

Doses of 2 mg. of these crystals were administered to each of two polyneuritic pigeons by intramuscular injection. No improvement ensued and both birds died within 24 hours.

Nitrogen determinations on both forms were made by the Kjeldahl-Gunning-Arnold method under conditions which have been found satisfactory for the determination of nitrogen in a variety of pyridine derivatives.[2] The results did not agree very satisfactorily, suggesting that the substances were not completely dried by the methods used. However, since more than 14 per cent of nitrogen was found in duplicate determinations upon

[2] Personal communication of Dr. E. K. Phelps and Mr. H. W. Daudt of the Bureau of Chemistry. These determinations were kindly made by Mr. Daudt.

each modification, it is apparent that neither is a hydrate. This is further shown by their interconvertibility in the absence of water.

It is impossible to predict accurately the onset of polyneuritis in birds or to keep them alive very long after the severe symptoms have appeared, and the preparation and preservation of pure and well defined crystal forms of α-hydroxypyridine are equally precarious undertakings; consequently the physiological tests of these substances presented many difficulties. However, the findings recorded in a previous paper (1) have been verified in every practicable way by curative tests, so that no doubt can remain that the antineuritic properties are associated only with the needles.

An attempt was made to conduct a protective experiment with the needle form on pigeons fed on white rice. The necessity of making repeated attempts to obtain small amounts of the pure needle form daily involved so much labor that the experiment was discontinued after 3 days. Four pigeons (Nos. 137 to 140, inclusive) were fed with white rice and given by mouth 5 mg. daily of the granular form. Within 3 days the birds were seen to be slightly stupid as compared with controls. On the 8th day the feathers of all the birds were ruffled and they stood in dejected attitudes. On the 9th day Nos. 137 and 139 from time to time were seized with violent attacks of vomiting. The pupils of the eyes were dilated, tremors were frequent, and breathing was slow and labored. The other two birds also showed mild tremors and dyspnea. The following day Nos. 137 and 139 were in a paralyzed condition indistinguishable from the acute type of polyneuritis, except that the birds at times continued to show the symptoms of the previous day. No. 139 died in this condition on the 10th day. No. 137 was treated with autolyzed yeast filtrate and on the following day was less affected by paralysis but remained in a semicomatose condition for 5 days and died without further notable change. After 11 days on white rice Birds 138 and 140 also showed some paresis as indicated by drooping of the wings and a tendency to stumble in walking. The administration of α-hydroxypyridine to the birds was discontinued and both the remaining pigeons temporarily improved, although the diet of white rice was continued. Bird 138 died

on the 23rd day with typical symptoms of polyneuritis and No. 140 on the 40th day with mild chronic paralysis and in a condition of extreme weakness. The rate of loss of weight in all these birds did not differ materially from that commonly observed in pigeons fed on white rice alone.

Measurements were made daily of the rectal temperatures of these birds from the 8th to the 14th day. The observations are shown in Table I.

TABLE I.

Rectal Temperatures (°C.) of Birds Fed on White Rice ad libitum + 5 Mg. Daily of the Granular Form of α-Hydroxypyridine.

Day of experiment.	No. 137.	No. 138.	No. 139.	No. 140.	Average of 6 controls.
8th	40.1	41.3	40.8	42.1	42.1
9th	38.9	41.3	40.6	41.7	
10th	39.9*	40.1	Dead.	41.3	42.2
11th**	40.8	39.4		40.1	41.8
12th	39.9	39.6		40.1	41.7
13th	40.8	40.8		40.3	
14th	Dead.	41.2		41.3	42.0

* Became sick and was treated with autolyzed yeast.

** Hydroxypyridine discontinued.

It will be observed that the temperature fell from day to day as long as the treatment with α-hydroxypyridine was continued, but later gradually rose again in the case of the two birds which continued on white rice alone as well as Bird 137 which was treated with autolyzed yeast filtrate. A progressive fall of rectal temperature has been observed in scores of birds after being fed on white rice in this laboratory for 15 days or more. The extent to which the temperature drops is variable, being greatest when the development of polyneuritis is retarded, and is attended by much emaciation. Although the rectal temperature of healthy pigeons also is variable, under laboratory conditions it is not far from 42°C. In contrast, a number of birds in a condition of chronic polyneuritis have been found to show rectal temperatures from 34–35° after 30 to 50 days on white rice. However, the development of early and sudden severe attacks of polyneuritis in pigeons which are not markedly emaciated also often is attended by a rapid fall of rectal temperature. Following successful

treatment with α-hydroxypyridine or with autolyzed yeast the temperature often rises 1–2° in the course of a few hours, but has not been seen to go above normal. Such observations were first made nearly 2 years ago, but no opportunity has arisen for making a study of the causes of the phenomenon, although it would seem to be worthy of thorough investigation. In this connection attention is called to the rises above normal temperature following successful treatment of human beri-beri, reported by Williams and Saleeby (26).

Whatever the significance of the rise and fall of temperature, it will be noted that such a fall appears to be accentuated by administration of the granular form of α-hydroxypyridine. The experiment above described would seem to add weight to the impression previously recorded that the granular form of α-hydroxypyridine is more detrimental to polyneuritic pigeons than to healthy ones. In any case it seems certain that α-hydroxypyridine ultimately exerts a toxic action since symptoms develop which are not commonly found in polyneuritis.

α-Methoxypyridine.—α-Methoxypyridine was prepared from α-hydroxypyridine according to the directions of Pechmann and Baltzer (2). The substance conformed to the description of these authors. Polyneuritic pigeons (Nos. 43, 44, 45, and 46) were treated by intramuscular injection of 5, 2, 2, and 0.5 mg. of α-methoxypyridine in aqueous suspension. All four birds were rapidly prostrated and in each case within 20 minutes were apparently insensible. No. 43 died after 80 minutes, No. 44 after 35, and No. 45 after 75 minutes. After a few hours No. 46 recovered partly from the earlier prostration and lived for 24 hours, but without any relief of the paralytic symptoms.

α-Methylpyridone.—This substance was first prepared from α-hydroxypyridine by the method of Pechmann and Baltzer (2). A portion of the product as liberated from water solution by saturation with potassium carbonate was used for treating polyneuritic pigeons Nos. 61 and 62. No improvement resulted and the birds died the following night. The remainder of the product was dried over calcium oxide and distilled (boiling point 250°). Polyneuritic pigeon No. 64 received an injection of a freshly made water solution of 4 mg. of this preparation. After 2 hours the bird was relieved of neck retraction and was able to

walk a few steps. It maintained a constant weight for 3 days and gained markedly in strength and activeness. On the 4th day paralysis redeveloped and death ensued on the 5th day.

Unfortunately an accident resulted in the loss of the remainder of this preparation of methylpyridone. A second quantity was prepared, by the method of Decker (8), by the oxidation of pyridine methyl iodide with potassium ferricyanide. The oil was dried over calcium oxide and distilled (boiling point 250°). In Table II the freshly dehydrated product is designated as "dehydrated." The oil was kept in a cork-stoppered tube for 4 months awaiting a convenient opportunity for further tests. In Table II this product is designated as "hydrated" since it was subsequently found to have absorbed moisture. A portion was again dried over calcium oxide and redistilled. It was used fresh and is designated "redistilled" in Table II, which contains a summary of the results of the physiological tests. In all cases a drop of the oil was weighed into a dry tube. A measured amount of cold water was added, the solution taken up in a syringe, and an aliquot injected intramuscularly within a few moments.

As will be noted the number of cases in which improvement occurred in birds treated with freshly dehydrated methylpyridone was five, as against two apparent failures. Only one bird, No. 148, could be said to have shown a change of condition at all comparable to that produced by autolyzed yeast. The remainder though distinctly improved remained dejected. No substantial increases in weight were noted in any of the birds.

Nine birds were fed on white rice *ad libitum* and in addition each daily received by mouth 2 mg. of methylpyridone in the form of a freshly prepared water solution. After 20 days the administration of methylpyridone was discontinued and all the birds remaining at that time temporarily improved markedly in general condition. It was evident that the general health of the birds was adversely affected by the treatment. Five birds, of which two had typical symptoms of polyneuritis, died in 14, 26, 28, 28, and 30 days respectively. The remaining 4 were alive but extremely emaciated at the end of 40 days, when the experiment was discontinued.

TABLE II.

Action of α-Methylpyridone on Polyneuritic Pigeons.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight	Treatment.	Post-treatment condition.	Length of life after treatment.
			per cent			*days*
75	42	Mild chronic paralysis, very weak.	44	5 mg. "dehydrated."	No change.	3
76	29	Severe paralysis, neck retraction.	38	5 " "	Neck not retracted; could stand but not walk next day.	3
101	26	"	35	5 " "hydrated."	Dead next day.	
104	20	Severe paralysis, intermittent neck retraction.	27	5 " "	Slightly improved.	5
105	20	Severe paralysis.	25	5 " "	Worse.	1
108	42	Completely paralyzed, very weak.	42	5 " "	"	2
117B		Severe paralysis.		5 " "redistilled."	Distinct improvement for 2 days. Otherwise treated after 4 days.	
148	20	Severe paralysis, neck retraction.	20	5 " "	Complete recovery from paralysis in 2 days.	8

TABLE II—*Concluded.*

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.	Post-treatment condition.	Length of life after treatment.
			per cent			*days*
173	30	Very weak and dejected, intermittent spastic attacks.	26	5 mg. "redistilled."	Free from spasticity next day; stronger and more vigorous on 2nd day.	6
200	24	Severe paralysis.	35	10 " "	Dead next day.	
207	24	"	34	10 " "	Intermittently spastic on 2nd day.	4
				5 " "	Still further improved on 3rd day. Two doses were given on successive days.	

Trigonelline.—Nicotinic acid methyl iodide was shaken in water solution with silver oxide (24) and the resulting trigonelline hydrate was shaken out with chloroform and crystallized from alcohol and then from water. On drying at 100° the hydrate lost one molecule of water. The hydrate was administered to polyneuritic pigeons Nos. 78 and 79 without result. Seven birds (Nos. 80, 81, 113, 162, 172, 192, 197) received injections of 2 to 10 mg. of the dehydrated trigonelline, administration being effected under the same conditions as in the case of methylpyridone. It seems unnecessary to tabulate the results in detail. They may be summarized as follows: No. 80, after a dose of 3 mg. recovered completely from paralysis in the course of 10 hours, remained lively and active for 3 days, and died on the 7th. Nos.

162 and 172 improved markedly under similar conditions but lived only 3 days, without recovering complete muscular control. The remainder showed no improvement. There was no gain in weight in any of the individuals, though No. 80 maintained constant weight for 4 days.

Six birds were fed on white rice and given by mouth 5 mg. each daily of dehydrated trigonelline. The birds died with evidences of polyneuritis in 27, 34, 41, 41, 46, and 46 days respectively. Life may have been prolonged somewhat, but otherwise the course of the disease did not appear to be materially altered by the treatment.

Betaine.—A quantity of betaine hydrochloride (Kahlbaum's) was treated with sufficient sodium carbonate to combine with the hydrochloric acid present. The solution was evaporated to dryness, the residue taken up in absolute alcohol, and the resulting free betaine recrystallized from alcohol and dehydrated at 110°. Table III shows the results obtained by the expeditious injection of freshly prepared water solutions of the dehydrated betaine. Only in one case did treatment with dehydrated betaine fail to effect substantial improvement, and there is reason to suspect that repetition of the treatment would have led to successful results in this case.

Eight birds were fed on white rice *ad libitum*. On alternate days each received an injection of 5 mg. of betaine in the form of a freshly prepared water solution. Definite polyneuritic symptoms appeared in one bird after 21 days in the form of mild lameness, which varied greatly from day to day in severity. Death followed 15 days after the first appearance of the symptoms. A second bird died with typical symptoms on the 34th day, while a third and fourth were found dead on the 34th and 37th days without having shown definite neuritis. The remaining four birds remained alive at the end of 42 days, when the experiment was discontinued.

Nicotinic Acid.—Nicotinic acid was prepared by the oxidation of nicotine with nitric acid in the usual manner (27). Such preparations of nicotinic acid purified by recrystallization from water and alcohol have been administered to a score or more of polyneuritic pigeons and fowls during the past 2 or 3 years, but in no case was there indisputable evidence of benefit. Such a

TABLE III.

Action of Dehydrated Betaine on Polyneuritic Pigeons.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.		Post-treatment condition.	Length of life after treatment.
			per cent	*day*	*mg.*		*days*
147	47	Severe paralysis, neck retracted.	40	1st 2nd 3rd	5 5 5	Little change on 2nd; improved on 3rd day and held head normally; no further change.	5
149	16	"	27	1st 4th	5 5	No change for 3 days. Nearly free from paralysis on 5th day; able to fly fairly strongly on 6th.	11
165	14	"	36		3	Nearly recovered on 2nd day; able to fly strongly on 3rd and so continued till the 11th day.	18
179	25	"	36	1st 3rd	5 5	Free from paralysis but stupid on 2nd day; worse on 3rd; better again on 4th. Increasing weakness throughout.	5
181	26	"	32		5	Completely cured of paralysis on 2nd day. No symptoms till 9th day.	14
183	16	"	33		3	No change on 2nd day.	3
196	22	"	25		5	Free from symptoms on 2nd day; returned on 9th.	11
198	21	"	23	1st 2nd 3rd	5 5 5	Not improved on 2nd day; spastic only at intervals on 3rd; fully recovered on 4th; symptoms returned on 12th.	13
216	29	Moderate paralysis, severely prostrated.	26	1st 3rd	5 5	Gradual improvement till the 5th day but never quite free from paralysis. Decline apparent on 10th day.	13

TABLE III—*Concluded.*

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Treatment.		Post-treatment condition	Length of life after treatment.
			per cent	*day*	*mg.*		*days*
229	28	Severe paralysis.	40	1st 2nd 4th	5 5 5	Improvement marked on 2nd day and continued till the 6th; remained somewhat ataxic. Gained 12 gm. in weight.	9
235	25	"	26	1st 3rd	5 5	Improved on 2nd day; free from paralysis on 4th; able to run freely on 6th but could not fly; declining on 8th.	13

preparation of nicotinic acid was sublimed and 3 mg. of the sublimate were administered to polyneuritic pigeon No. 74 without result. Pigeon 199 received an injection of 3 mg. of nicotinic acid which had been continuously subjected to a temperature of 140° for 6 days. No improvement resulted. Pigeon 77 received without benefit 5 mg. of the substance after it had been heated in a sealed tube at 250° for 3 hours. The following day this bird was treated by another method. A small amount of nicotinic acid was converted into the hydrochloride. This was decomposed in water solution, with freshly precipitated silver oxide, and the filtrate from the silver chloride was injected. Within 3 hours the bird was standing in a nearly normal attitude although it had been suffering from severe neck retraction when observed at frequent intervals for 24 hours previous. Nevertheless the pigeon was again completely paralyzed on the following day. It then received the filtered solution resulting from the decomposition by hydrogen sulfide of a few mg. of silver nicotinate suspended in water. No improvement ensued and the bird was dead the next morning. Pigeons 192 and 194 also were treated with the last mentioned product without result.

TABLE IV.

Effect on Polyneuritic Pigeons of Solutions Resulting from the Decomposition of Nicotinic Acid Hydriode with Silver Oxide.

Bird No.	Days fed on white rice before onset.	Symptoms.	Loss in weight.	Dose equivalent to nicotinic acid.	Post-treatment condition.	Length of life after treatment.
			per cent	mg.		days
143	32	Severe paralysis and neck retraction.	30	5	Assumed natural attitude after 3 hrs. Spastic at intervals. Still occasionally spastic next day. Free from paralysis on 2nd day but still weak. Weight constant for 5 days.	10
152	30	"	32	2	Distinct relief in 2 hrs. Little further improvement on 2nd day. Entirely free from paralysis on 3rd and able to fly. Gained 20 gm. in weight. Put on a diet of wheat on 7th day.	Still living and well.
155	33	General prostration.	46	5	Died during the night.	
193	16	Severe paralysis and neck retraction.	26	5	Nearly free from paralysis in 12 hrs. Able to fly strongly on 2nd day. Lively and vigorous. Gained 12 gm. in weight. Symptoms returned on 5th day.	5

Following Kirpal's method for converting nicotinic acid methyl iodide into trigonelline (24), nicotinic acid hydriodide was decomposed with silver oxide. Care was taken to avoid the use of any considerable excess of silver oxide. The fresh filtrate from the resulting silver iodide was injected promptly. Table IV shows the physiological results obtained. The rapidity with which the paralysis was relieved in Birds 143 and 152 is noteworthy.

SUMMARY.

1. The two crystalline forms of α-hydroxypyridine are isomers and mutually convertible into one another.

2. Only the needle form of this substance is curative for polyneuritis. The corresponding crystal form of β-hydroxypyridine and the anhydrous forms of methylpyridone, trigonelline, and betaine produce similar curative effects on polyneuritic birds. The last three named have been tested for protective properties with negative results.

3. It is concluded that the curative form of α-hydroxypyridine is a pseudo betaine and that a feature conforming more or less closely in structure or energy conditions to the type of a betaine ring is probably an essential characteristic of antineuritic "vitamines."

4. Attention is called to the theoretical possibility of the existence of such a structure in most of the simpler nitrogenous constituents of animal tissues, but especially in the nuclein bases.

6. Preliminary experiments are described which suggest that nicotinic acid may exist in a betaine form and that the curative properties of Funk's "vitamine" fractions of yeast and rice polishings may have been due in part to this isomeric form of nicotinic acid or a polymer or simple derivative of it.

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EXPLANATION OF PLATE 4.

FIG. 1. A microscopic field of the freshly prepared needle form of α-hydroxypyridine contaminated with a few granular crystals, the material being mounted in a benzene-ligroin mixture.

FIG. 2. The same microscopic field reproduced as it appeared after the lapse of 30 minutes. Note the growth of the granular form at points of original contamination. Further transformation has been retarded by the spontaneous evaporation of the film of liquid, the receding edge of which is seen in the upper photograph.

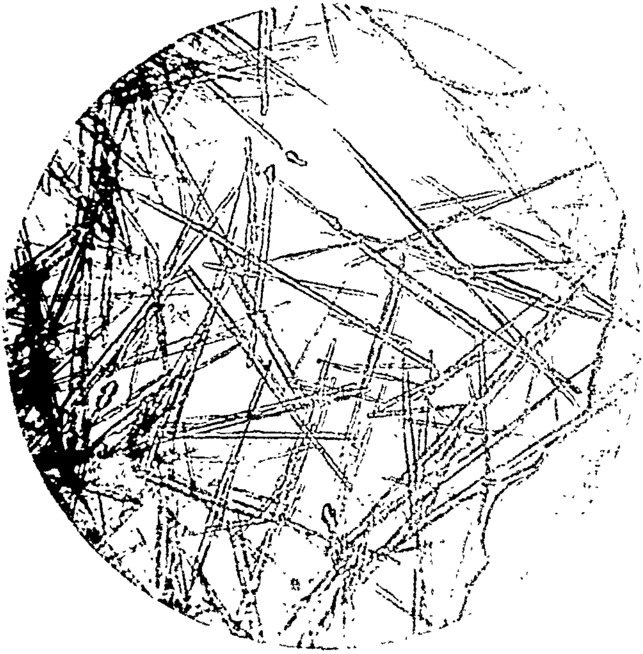

Fig. 1.

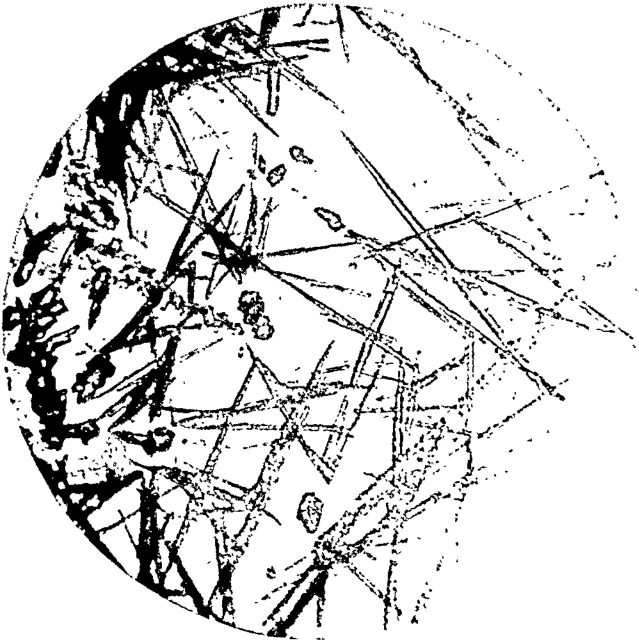

Fig. 2.

THE DIETARY DEFICIENCIES OF THE WHITE BEAN, PHASEOLUS VULGARIS.*

By E. V. McCOLLUM, N. SIMMONDS, and W. PITZ.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

Plate 5.

(Received for publication, February 27, 1917.)

Preliminary to our plan for making an elaborate inquiry into the supplementary dietary relationships among our natural foodstuffs, we have carried out a series of feeding experiments with a long list of the individual natural foods. We have supplemented these with single and multiple additions of purified food factors, *e.g.*, protein, inorganic salt mixtures of appropriate composition, fat-soluble A, and water-soluble B, in order to evaluate these several dietary factors in each of the natural products which we are studying in combinations. We have described in previous papers the results obtained by this procedure with wheat,[1] rice,[2] wheat germ,[3] maize,[4] and oat kernel.[5]

In the present paper we describe the behavior of young rats when fed the white bean of commerce as the principal constituent of the diet, and supplemented with purified food factors in various ways. The results indicate clearly the low biologic value

* Published with the permission of the Director of the Wisconsin Experiment Station.

[1] Hart, E. B., and McCollum, E. V., *J. Biol. Chem.*, 1914, xix, 373.
McCollum, E. V., and Davis, M., *ibid.*, 1915, xxi, 615.
Hart, E. B., Miller, W. S., and McCollum, E. V., *ibid*, 1916, xxv, 239.
McCollum, E. V., Simmonds, N., and Pitz, W., *ibid.*, 1916–17, xxviii, 211.

[2] McCollum and Davis, *J. Biol. Chem.*, 1915, xxiii, 181.

[3] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916, xxv, 105.

[4] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 153.

[5] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1917, xxix, 341.

of the proteins of the bean when fed as the sole source of amino-acids.

Because of the high content of protein in beans, they, or more generally the legumes as a class, have been frequently recommended as substitutes for the apparently more expensive sources of protein; *viz.*, meat, milk, and eggs. It is clearly apparent that such advice may be misleading, since modern investigations of proteins by chemical and biologic methods have made it clear that from the dietetic standpoint the term protein cannot be loosely employed as it was formerly. Protein is of biologic value only in so far as it yields appropriate proportions of the essential amino-acids. The facts brought out by the data here presented may be summarized as follows.

1. The low nutritive value of the bean proteins is shown by the behavior of Lot 718 (Chart 1) whose ration contained 19.8 per cent of proteins all derived from the bean. There was high mortality in animals fed this ration, and pronounced stunting was observed in all.

Lot 830 (Chart 2) whose ration contained 19.0 per cent of protein from the bean and 3 per cent of casein was noticeably improved as the sole result of the small casein addition. It is evident from the results shown in Charts 1 and 2 that there is only a moderately efficient supplementing action of bean proteins by casein. The improvement in Lot 830 over Lot 718 seems to be due to the *additional* protein as casein in greater measure than to a marked increase in the value of the beans through the supplying of a particular amino-acid present in very small amount in bean proteins and plentiful in casein.

Lot 720 (Chart 3) and Lot 721 (Chart 4) serve further to support the view expressed above regarding the low biologic value of the bean proteins. Our experience with the group of proteins contained in the bean confirms the experience of Osborne and Mendel[6] with phaseolin, the principal protein of the bean, which they isolated and fed in purified form supplemented with "protein-free milk" as a source of the unidentified dietary factors and inorganic salts. They found phaseolin to possess very little biologic value.

[6] Osborne, T. B., and Mendel, L. B., *Z. physiol. Chem.*, 1912, lxxx, 307.

2. A high content of beans in the diet appears to exert an injurious effect on the rat. This is illustrated by the history of Lot 720 (Chart 3) as compared with that of Lot 621 (Chart 5). The only significant difference in the rations of these two groups was in the content of beans. The former contained 70 per cent of beans and the latter 45 per cent. Each ration contained 9 per cent of casein. The animals fed the lower content of beans (Lot 621) and consequently the lower protein supply were much better nourished than were those in Lot 720. The same is true of Lot 719 (Chart 6) with a ration containing 50 per cent of beans with 4 per cent of casein as contrasted with Lot 830 (Chart 2) whose ration contained 86.3 per cent of beans and 3 per cent of casein. The latter with 19 per cent of protein were not so well nourished as the former with but 15 per cent. The young which were successfully weaned in Lot 621, with the lower content of beans, were able to grow normally to full adult size and produce young. Those which were reared on Ration 720 with the higher bean content were able to grow at less than half the normal rate (compare Charts 3 and 5). Two explanations seem sufficiently plausible to warrant brief comment. It may be that beans contain some unknown chemical complex which is harmful and that young rats can tolerate without pronounced injury the intake of the hypothetical substance carried by the lower plane of intake of beans, but show the effects of intensifying this factor. Provisionally, however, we have adopted the view that the rats are mechanically injured by the distention of the digestive tract, due to the extent of fermentation processes after eating liberally of beans. The latter contain much carbohydrate of the class of the hemicelluloses, for the digestion of which the higher animals have no provision. These hemicelluloses are, however, readily attacked by microorganisms inhabiting the digestive tract, and their fermentative decomposition is accompanied by the liberation of a great deal of gas. That this is in great measure responsible for the injurious effects following high bean feeding is supported by the marked tympanites observed regularly in experimental animals eating rations high in beans (Figs. 1 and 2). In rats under 45 gm. body weight the distention of the intestinal tract with gas appears to be the direct cause of death on such rations. Partial asphyxiation of

the walls of the intestinal tract through interferences of the circulation caused by excessive stretching is, we believe, the principal factor in debilitating the animals, but the low quality of the proteins is in some of the rations here described a contributing factor.

3. *The white bean appears to be even poorer in its content of the dietary factor fat-soluble A than are the cereal grains.* This is illustrated by the records of Lot 611 (Chart 7) whose ration in Period 1 was closely similar to that of Lot 621 (Chart 5), except that no butter fat was included. During a period of 8 weeks there was no growth on this ration, but the animals responded at once with growth at a good rate when in the second period 5 per cent of butter fat replaced its equivalent of beans.

4. *The inorganic content of the bean is not of a character which supports growth.* This is seen in the records of Lot 778 (Chart 8) whose ration closely resembled that of Lot 621 (Chart 5) except that no salt mixture was added in Period 1. No growth took place until in the second period 1.90 per cent of a suitably constituted salt mixture was introduced, when the animals at once responded with increase in body weight. In this respect the bean behaves like all other seeds which we have studied. In all cases the seeds of plants require a modification of their inorganic content by salt additions before growth can take place when the other dietary factors are suitably improved by the addition of purified food substances. With combinations of seed with leaf we have been able to nourish rats adequately through four generations on a strictly vegetarian diet without either protein, salt, or fat-soluble A additions.[7] This is possible because of the high content and peculiar composition of the inorganic content of the leaf[8] and its richness in the fat-soluble A.

5. *The richness of the bean in the unidentified water-soluble B* is shown by the remarkable history of Lot 696 (Chart 9) whose ration consisted of a mixture of purified foodstuffs including 5 per cent of butter fat to furnish the fat-soluble A. The ration was so composed as to require only the addition of the water-soluble B to support growth. The sole function of the 25 per cent of beans in the food mixture was to furnish this dietary factor. On this ration growth was complete and one female has produced two

[7] McCollum, Simmonds, and Pitz, *Am. J. Physiol.*, 1916, xli, 333.

[8] Forbes, E. B., *Ohio Agric. Exp. Station Bull. 222*, 1910.

litters of young (eighteen young) of which eight were reared. The beans employed in this ration were soaked in distilled water and heated for $1\frac{1}{4}$ hours at 15 pounds' pressure, dried, and ground.

Mendel and Fine[9] noted the relatively low utilization of the proteins of the bean and showed that the cause lay in part in the high content of indigestible carbohydrate. This prevents digestion to some extent by protecting the proteins from contact with the digestive juices. The indigestible carbohydrate likewise acts as a sponge to absorb the secretions of the tract with their content of proteins and their cleavage products, and prevents their reabsorption.[10] Our own experiments furnish conclusive evidence that the entire protein mixture of the bean is of low value, but further inquiry must be made to show which amino-acid is the limiting factor in determining the poor quality of these proteins.

Goldberger[11] has emphasized the desirability, in planning dietaries for the treatment of pellagra, or for the prevention of its recurrence, of including fresh animal and leguminous protein foods. It is, of course, probable that properly planned experimental inquiry will show that when combined with appropriate proportions of the protein of certain other natural foods, the proteins of the bean may be greatly enhanced in biologic value. We have under way an investigation of this question. It should be emphasized that the evidence at present available points to the conclusion that the bean proteins are decidedly inferior to those of meat, milk, eggs, and the cereal grains which have thus far been carefully studied, *viz.*, wheat, maize, oat, and rice kernels.

The bean contains an abundance of the unidentified water-soluble B, since 25 per cent of beans as the sole source of this factor (Chart 9) supports completion of growth and repetition of reproduction. In this respect it does not differ markedly from the cereal grains.[12] We would point out, therefore, that by the methods of investigation we have employed, no peculiar dietary properties of the bean indicating unusual value have

[9] Mendel, L. B., and Fine, M. S., *J. Biol. Chem.*, 1911–12, x, 433.
[10] Mendel, L. B., and Lewis, R. C., *J. Biol. Chem.*, 1913–14, xvi, 55.
[11] Goldberger, J., *J. Am. Med. Assn.*, 1916, lxvi, 471.
[12] McCollum, Simmonds, and Pitz, *J. Biol. Chem.*, 1916–17, xxviii, 229.

been revealed. Unless, as has been pointed out above, it shall be found that proteins from other constituents of the diet make good the chemical deficiencies of the proteins of the bean, the generalization of McKay[13] that they (the legumes) "as a source of protein are superior to most of the cereals" cannot be accepted in the light of experimental work now available. Judging from the decided damage to the intestine which results from the excessive fermentation of the carbohydrates of beans we feel safe in saying that this legume should under no circumstances form a principal part in any relatively monotonous diet such as many institutions even in this country furnish their inmates.

Finally, mention should again be made of the fact that our simple interpretation of the causes of failure in the case of certain of our diets proves adequate in all cases. The data furnished in this and our former papers[1-5] now warrant the conclusion that the great problem in practical dietetics and in animal production lies not in securing enough of the unidentified chemical complex associated with the causation of polyneuritis (water-soluble B), but in securing a satisfactory adjustment among (1) the digestion products of the proteins of the diet (amino-acids), (2) an adequate supply of the fat-soluble A, and (3) more important even than a high biologic value of the protein mixture of the ration, a content of the essential inorganic elements suitable in amounts and proportions.

Our extensive experience in feeding natural food substances restricted to a single source now leaves no room for doubt that the character of the inorganic content of the food mixture if fed monotonously constitutes a factor of the greatest importance in influencing growth and well-being. Furthermore, it is now evident that so small an amount as 15 per cent of wheat kernel, 3 per cent of wheat germ, or 25 per cent of thoroughly cooked beans serve to supply an ample amount of all the as yet chemically unidentified food essentials except that supplied by butter fat (fat-soluble A). We are convinced that the prevalent tendency to regard several types of malnutrition (scurvy, pellagra, rickets, etc.) as being each due to the lack of a specific chemical complex in the diet cannot be supported by convincing experimental proof. Any diet containing a moderate content of a wholesome natural food will not be lacking in the water-soluble B.

[13] McKay, D., The Protein Element in Nutrition, London, 1912, p. 58.

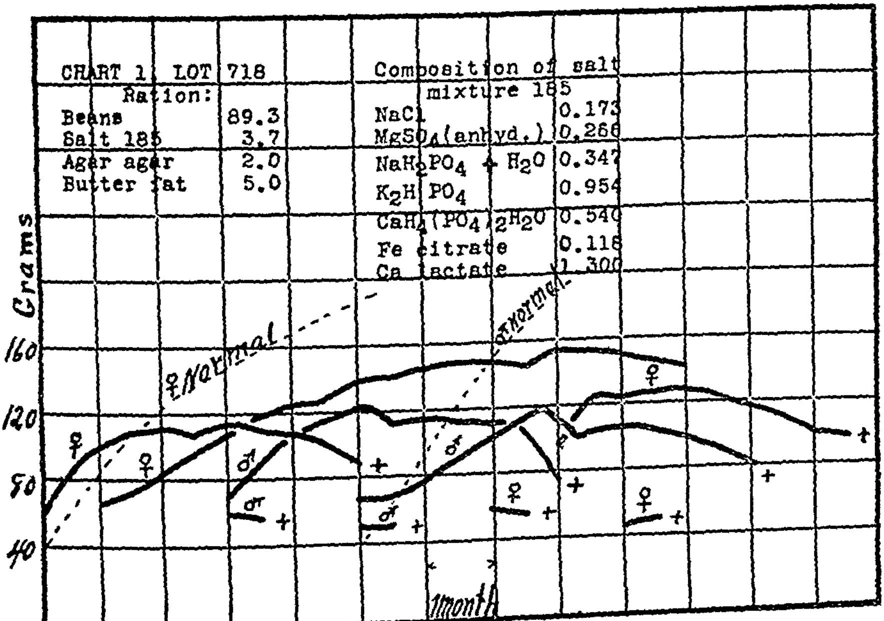

CHART 1. Lot 718 illustrates the poor growth of rats fed a diet which as far as concerns chemical complexes is an adequate food mixture except for the poor quality of the proteins, all of which (19.6 per cent) are derived from beans. The validity of this explanation is evident from the records of Lot 830, Chart 2, and of Lot 720, Chart 3, whose rations were closely similar to those of 718, Chart 1, except that 3 per cent and 9 per cent of casein respectively were included in the diets. Four younger animals failed entirely to grow on this diet and died after 2 to 3 weeks. We attribute a positive injurious effect of a high bean content in a monotonous diet to the excessive fermentation of hemicelluloses.

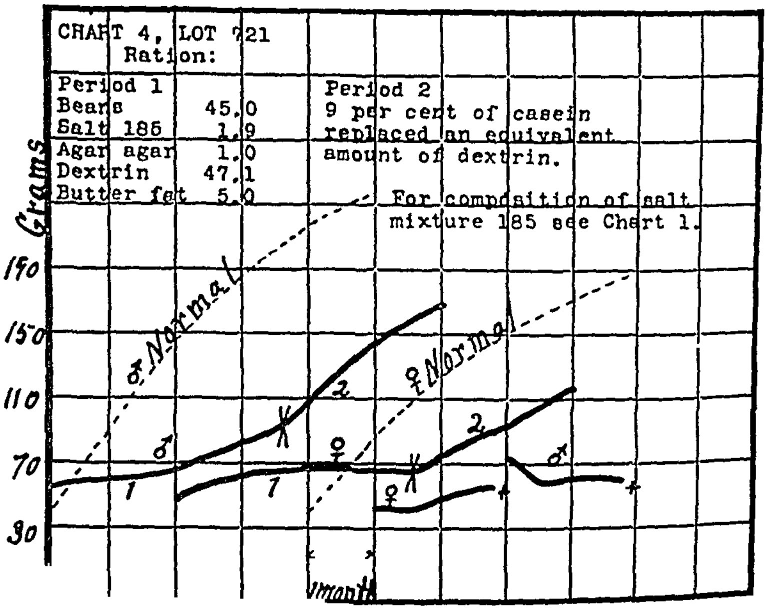

CHART 4. Lot 721 was introduced into the series of experiments here described to ascertain whether by reducing the content of beans, and therefore the amount of indigestible but fermentable carbohydrates (hemicelluloses), the animals might make better use of the proteins of the bean. This did not prove to be the case. After 3½ months of suspended growth they responded promptly when 9 per cent of casein was introduced into the diet. Compare these records with those of Lot 621, Chart 5, whose diet contained 9 per cent of casein throughout the experiment but was otherwise identical with that of Lot 721.

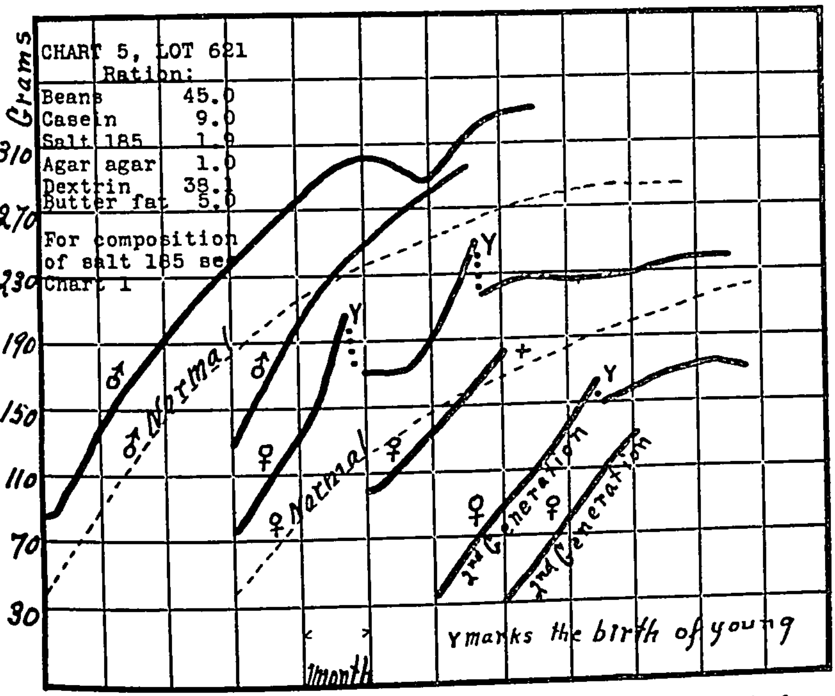

CHART 5. Lot 621. These records, when considered along with those of Chart 4, Lot 721, indicate clearly that the factor which is responsible for the poor nutrition of the latter was the poor quality of the proteins of the beans. That the content of the beans in easily fermentable hemicelluloses, for the digestion of which the higher animals are not provided with enzymes, is a depressing factor in our experiments containing beans is emphasized by a comparison of Chart 5 with Chart 3, Lot 720, whose ration was closely similar but contained 70 per cent of beans. The rats receiving the lower content of total protein (and hemicellulose) were distinctly better nourished than those receiving the higher bean allowance. This we attribute to the mechanical injury resulting from distention of the digestive tract with gas. Ration 621 contained 18.9 per cent, and Ration 720 24.4 per cent of protein.

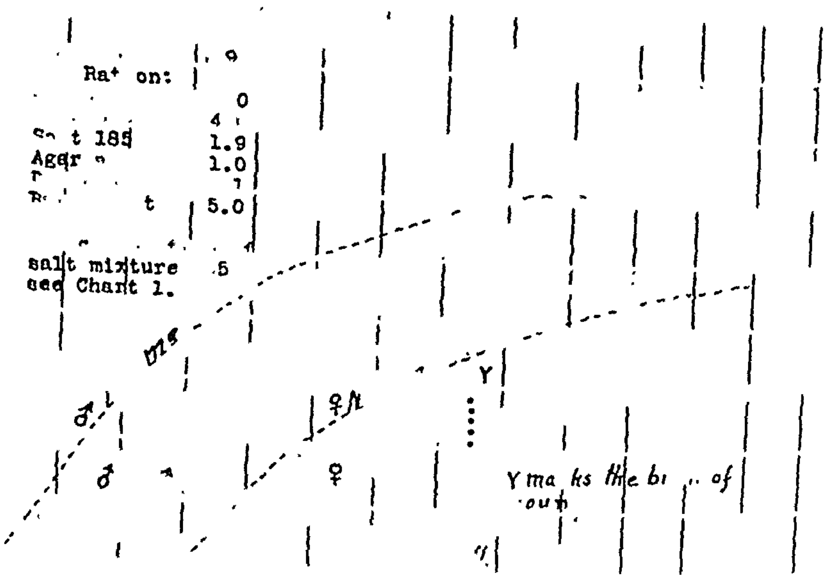

CHART 6. Lot 719 illustrates the character of the growth curves obtained with a diet all factors of which were fairly satisfactorily constituted but which derived its protein (15 per cent) from 50 per cent of beans and 4 per cent of casein. Growth was somewhat below normal and reproduction greatly depressed. No young were reared. Poor quality of the protein moiety and the depressing effects of excessive intestinal fermentation are the causes of the unsatisfactory nutrition of these rats. Compare with Lot 830, Chart 2, and Lot 621, Chart 5.

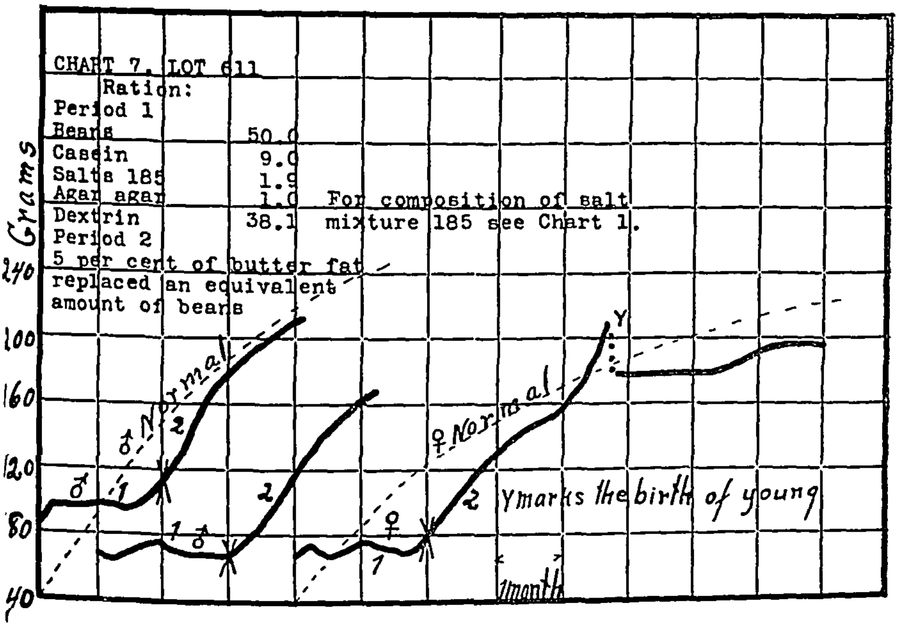

CHART 7. Lot 611 shows that beans contain very little, if any, of the unidentified fat-soluble A. In Period 1 there was no growth, although the diet was adequate except for its lack of the fat-soluble A. After 2 months' suspension of growth the animals responded with normal growth on the inclusion in Period 2 of 5 per cent of butter fat in the diet. This diet contained 20 per cent of protein, 11 per cent of which was furnished by beans.

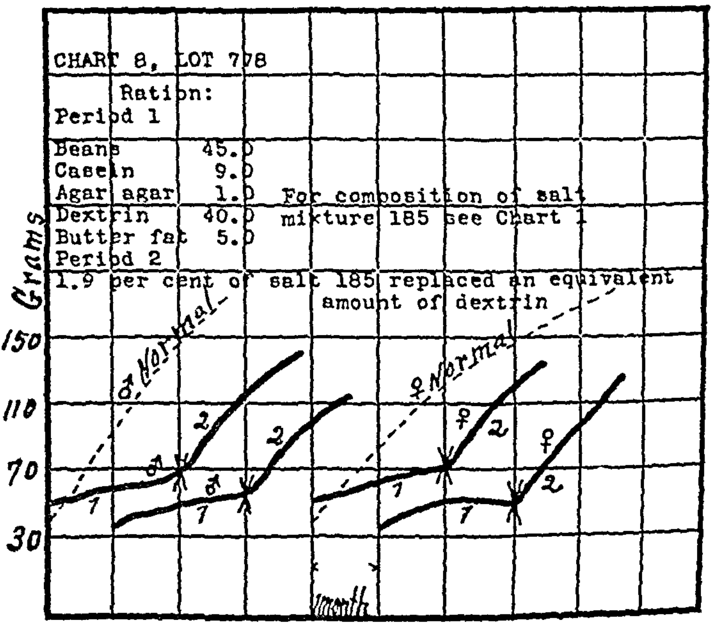

CHART 8. Lot 778 illustrates the fact that the inorganic content of 45 per cent of beans, supplemented with the phosphorus and sulfur in 9 per cent of casein does not support growth even though the organic portion of the ration is adequate for fairly good nutrition over a long period (compare Chart 5, Lot 621). In Period 2 the addition of 1.9 per cent of Salt Mixture 185 induced prompt resumption of growth at the normal rate. The bean, like the cereal grains we have studied,[1–5] contains an inorganic content which is unsatisfactory in its content and composition for the promotion of physiologic well-being in a growing animal.

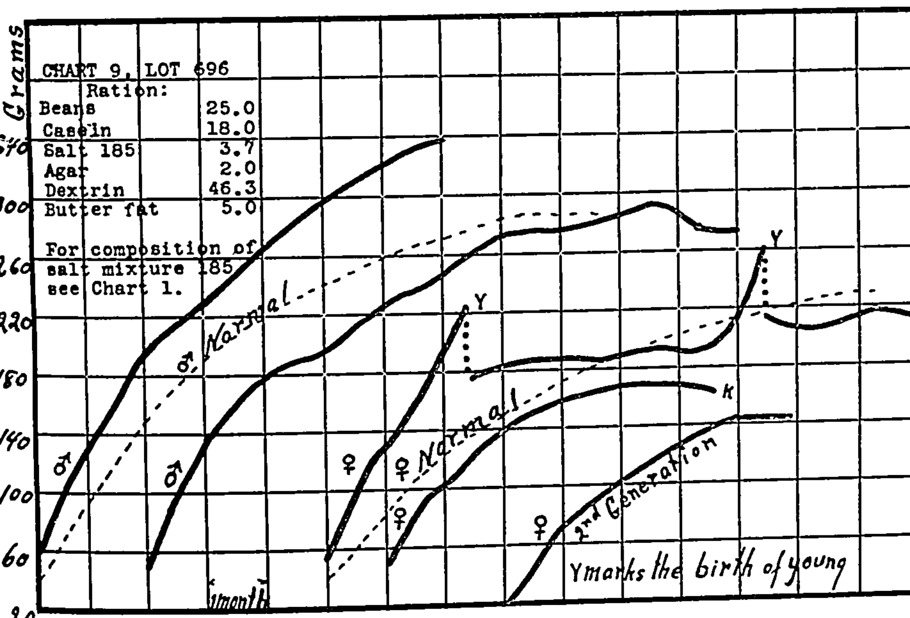

Chart 9. Lot 696 shows the richness of the bean in the unidentified dietary factor water soluble B. This ration, aside from its bean content, consists of a mixture of purified food substances which supplies adequate protein, salts, carbohydrate, and fat-soluble A (in the butter fat). The sole essential function of the 25 per cent of beans in the food mixture is to furnish the water-soluble B. The beans were soaked in water and then heated for 1 hours in an autoclave at 15 pounds' pressure, dried, and ground before incorporation with the ration. Even after this drastic treatment, beans to the extent of one-fourth of the food mixture supplied enough of the factor B to induce perfectly normal growth. One female produced two litters of young (eighteen young), of which eight were successfully weaned. The curve of one of her daughters is practically normal on this ration.

EXPLANATION OF PLATE 5.

Fig. 1. The appearance of the abdomen of a normal rat.

Fig. 2. The appearance of the abdomen of a rat fed a monotonous ration containing a high content of beans. Note the marked tympanites.

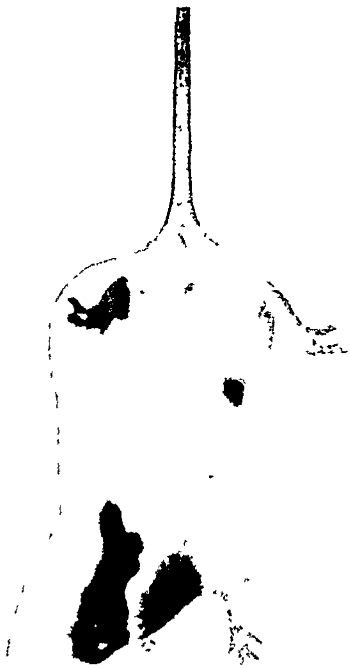

FIG. 1.

FIG. 2.

(McCollum, Simmonds, and Pitz: Deficiencies of White Bean)

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PROCEEDINGS OF THE AMERICAN SOCIETY OF BIOLOGICAL CHEMISTS.

ELEVENTH ANNUAL MEETING.

New York, N. Y., December 28–30, 1916.

THE DIASTATIC ACTIVITY OF THE BLOOD IN DIABETES.

By VICTOR C. MYERS and JOHN A. KILLIAN.

(From the Laboratory of Pathological Chemistry, New York Post-Graduate Medical School and Hospital, New York.)

Although it has been recognized since the time of Magendie that blood possesses active diastatic properties, no studies of a conclusive nature appear to have been published relative to the activity of the blood diastase in human subjects suffering from diabetes. This has probably been due to the lack of a sufficiently delicate and accurate method.

It has been found that the procedure introduced by Lewis and Benedict[1] for the estimation of the sugar of the blood, may be excellently utilized in the estimation of its diastatic activity. Two 2 cc. samples of oxalated blood are taken, one being employed as a control. The control tube is made up to 10 cc. with distilled water, and the tube to be employed for the test to 9 cc. Both tubes are now placed in a water bath at 40°C. As soon as the contents of the tubes have been brought to this temperature, 1 cc. of 1 per cent soluble starch is added to the second tube, the contents are mixed, and incubation is then carried out for exactly 15 minutes at 40°C.

After the incubation has been completed, about 1.0 gm. of dry picric acid is at once added to each tube and the mixtures are stirred. When the proteins are precipitated, the tubes are centrifuged and the supernatant fluid is filtered. The sugar in 3 cc. portions of the filtrates is now estimated according to the technique described by Myers and Bailey.[2] Correction is made for the sugar originally present in the blood (with the aid of the control) and for the slight reducing action of the soluble starch. The results have been recorded in terms of the percentage of the soluble starch (10 mg.) transformed to reducing sugars (calculated as glucose) by the 2 cc. of blood employed.

[1] Lewis, R. C., and Benedict, S. R., *J. Biol. Chem.*, 1915, xx, 61.
[2] Myers, V. C., and Bailey, C. V., *J. Biol. Chem.*, 1916, xxiv, 147.

It is believed that 10 mg. of starch furnish a sufficient substrate for all ordinary conditions with human blood (except possibly in very severe diabetes), since practically identical results have been obtained with amounts of starch up to 40 mg. Glycogen offers no advantage over soluble starch, since the results are essentially identical. The possible error of glycolysis would appear to be a negligible one during a 15 minute period of incubation.

The diastatic activity of the blood, with this method, has been found to vary from 15 to 25 in a variety of miscellaneous conditions in the human subject. In cases of advanced nephritis with nitrogen retention and hyperglycemia, figures from 30 to 40 have been observed, while in eleven cases of diabetes the values ranged from 40 to 74. These results would suggest that the increased diastatic activity in both nephritis and diabetes (as shown by the analyses of the blood) may be the important factor in the production of the hyperglycemia in these conditions.

THE BLOOD LIPOIDS IN NEPHRITIS.

By W. R. BLOOR.

Lipemia has occasionally been reported in nephritis, and because of its importance in the study of fat metabolism an examination of the blood lipoids in a series of cases of severe nephritis was undertaken. The results showed mainly marked increases in the total fatty acids (due to fat) in the blood plasma and of lecithin in the corpuscles, with less marked and less frequent increases of fat and of cholesterol in the corpuscles. These abnormalities are essentially those found in the blood in alimentary lipemia and their presence in nephritis is believed to be due to a retarded fat assimilation which is one manifestation of a general phenomenon produced by the acidosis present in this condition.

THE LIPOIDS OF THE BLOOD IN RENAL CONDITIONS, WITH SPECIAL REFERENCE TO THE CHOLESTEROL CONTENT.

By ALBERT A. EPSTEIN AND MARCUS A. ROTHSCHILD.

By means of the newly developed methods for the examination of fats and lipoids in the blood, it has been possible for us to examine nearly 100 cases of renal disease and allied conditions.

The results may be briefly summarized as follows: In chronic parenchymatous nephritis (nephrosis), particularly in the edematous stage, the lipoids of the blood are exceedingly high; the cholesterol content, to which special attention has been given in this study, has been observed as high as 1.226 gm. per 100 cc. In other renal conditions, including surgical cases, no such increase has been encountered. Occasionally a moderate rise has been found, but as a rule this was associated with some other superimposed condition.

In uremic cases, particularly those showing high nitrogen retention in the blood, the lipoid content is very much diminished. The cholesterol in such cases may fall as low as 0.080 gm. per 100 cc. Several effusion fluids from cases of parenchymatous nephritis have been examined and only traces of lipoid have been found in them. The urines of these cases, when rich in protein material, show the presence of small amounts of lipoid.

The origin of the increase of the lipoid content of the blood in the parenchymatous form of nephritis is not clear. Undoubtedly a large portion, perhaps the greatest, is due to ingested lipoid material. A certain part may be ascribed to mobilization of body fat, particularly as these cases when recuperating show extreme emaciation. This high grade form of malnutrition is undoubtedly associated with considerable tissue degeneration, so that a certain portion of the lipoid in the blood must be attributed to that source. At any rate, the finding undoubtedly represents a condition of fat non-utilization, comparable to that found in diabetes and other nutritional disorders. This is evidenced also by the fact that when a diet consisting of large amounts of protein, a small amount of carbohydrate, and practically no fat, is administered for a long period of time, the excessive lipemia and lipoidemia gradually subside.

OBSERVATIONS ON ACID-BASE EQUILIBRIUM IN THE BODY.

By JOHN HOWLAND and W. McKIM MARRIOTT.

(From the Harriet Lane Home and Department of Pediatrics, Johns Hopkins University, Baltimore.)

Known amounts of hydrochloric acid, of acid sodium phosphate, and of sodium phosphate of blood reaction were administered to normal adults. Determinations on the urine showed

that hydrochloric acid administration led to an increase in titratable acid (A) and a proportionate increase in ammonia excretion, the ratio A:NH_3 remaining constant. Acid sodium phosphate increased the titratable acid excretion but had no effect on ammonia excretion. The ratio A:NH_3 was greatly increased. Administration of phosphate mixtures of blood reaction (P_H=7.4) led to a slight increase in the titratable acid and to a distinct diminution in ammonia excretion. The A:NH_3 ratio was considerably above normal limits.

These findings may be taken to explain, in part, at least, the low excretion of ammonia in the acidosis of nephritis, it having been previously demonstrated that this condition is accompanied by a retention of acid phosphate.

ADENINE AND GUANINE IN COWS' MILK.

By CARL VOEGTLIN and C. P. SHERWIN.

(*From the Hygienic Laboratory, United States Public Health Service, Washington.*)

From 100 liters of a mixed sample of cows' milk it was possible to isolate 500 mg. of adenine and 100 mg. of guanine by means of silver precipitation of the protein-free milk residue. These findings therefore prove the existence of two hitherto unknown constituents of milk.

THE ANABOLIC ACTION OF THE THYROID GLAND.

By N. W. JANNEY.

(*From the Chemical Laboratory of the Montefiore Home and Hospital, New York.*)

The catabolic action of the thyroid, as exemplified in the treatment of obesity, takes place when large doses of thyroid preparations are administered. It was studied by us in nitrogen partition experiments made on fasting dogs. A greatly increased excretion of all the nitrogenous urinary constituents, except creatinine, took place, indicating a stimulation of catabolic cellular metabolism. Fever and other toxic symptoms were likewise present. The anabolic action of the thyroid has remained obscure, as it can be demonstrated only by giving very

small doses of the gland or its preparations. The results in many respects are the opposite of the catabolic and toxic action.

Using the nitrogen balance as a gauge of the effect of thyroid treatment, we have studied the results of administering small doses of thyroid preparations to cretins and exophthalmic goiter patients over extended periods. It could be shown that an added retention of nitrogen accompanied improvement in the clinical symptoms.

The same amounts of thyroid preparations fed to normal control persons on the same diet and under the same conditions were followed either by no change or by a reduction in the nitrogen balance.

In other preliminary experiments evidence has been obtained that the anabolic action which the thyroid gland exerts on protein metabolism is connected with its effect on carbohydrate metabolism. Thus the protein-sparing action of sugar which was found to be greatly reduced after thyroidectomy could be restored by feeding in addition small doses of thyroid.

ENZYME AND SUBSTRATE IN AUTOLYSIS.

By MAX MORSE.

The following reasons may be given for believing that the effect of acid introduced or already present in an autolyzing digest is upon the enzyme, and that the effect upon substrate, discovered by Bradley, is of secondary and not primary importance.

1. All known enzymes respond to alterations in hydrogen ion concentration. Therefore, if the effect of acid is upon the substrate from the inception of autolysis, as Bradley believes, no provision is made for this response and one must conclude that the enzyme is refractory to modification of the concentration of hydrogen ions. In this case the enzyme differs from all known ferments.

2. The substrate theory demands a high degree of specificity on the part of the enzyme, owing to the belief that the amount of substrate available for the enzyme is conditioned by the concentration of hydrogen ions. At the same time, Bradley has shown that the enzyme is not specific, since foreign proteins (casein, coagulated proteins, etc.) are digested.

3. The addition of coagulated proteins to a digest together with the addition of acid does not lead to acceleration of autolysis. This is interpreted on the substrate theory by assuming that there is no further activation of the enzyme. It is equally well interpreted as meaning that no more enzyme is introduced, for on introducing enzyme the rate is increased.

4. Inorganic colloids accelerate autolysis (Ascoli and Izar). Pincussohn, Wohlgemuth, and others have demonstrated an effect upon salivary amylase, pepsin, etc., when subjected to various inorganic suspensoids, so it is probable that a similar effect is exerted upon the autolytic enzyme.

5. Calcium salts, such as phosphate, sulfate, etc., show no acceleration in autolysis when used in hydrogen equivalent solutions, while similar salts of potassium, sodium, etc., show marked acceleration. The "salt effect" of Loeb is doubtless operative here and we have a basis for the suggestion herein made that the effect of acid is first to modify the permeability of the membranes and secondly to activate the enzyme.

ANTAGONISTIC ELECTROLYTES AND JELLY FORMATION.

By G. H. A. CLOWES.

When NaCl is added in increasing proportion to aqueous suspensions of soaps or lipoids, the dispersion of the particles in water is at first promoted, reaches an optimum at approximately 0.15 M NaCl, which corresponds approximately with the concentration of this substance in the blood, as noted by Koch. Subsequently an aggregating effect is exerted, precipitation occurring at a concentration of 0.35 to 0.4 M NaCl, which appears to be the limiting concentration for the maintenance of life of marine organisms, of mice on intravenous injection, and the precipitation point for emulsions, jellies, and a variety of other physical systems.

Koch's curve of the amount of $CaCl_2$ required to precipitate lecithin at different concentrations of NaCl corresponds closely with curves obtained by the drop method[3] when NaCl is added in increasing proportion to an aqueous phase containing a constant amount of soap passed through neutral oil. The initial

[3] Clowes, G. H. A., *J. Physic. Chem.*, 1916, xx, 407.

rise in drop number attributable to preferential adsorption of Cl ions promoting dispersion of the soap particles reaches an optimum at 0.15 to 0.25 M and subsequently diminishes, presumably owing to the relatively increased adsorption of the Na ion with rise in concentration. Soap jellies may be obtained at concentrations between 0.25 and 0.4 M NaCl provided sufficient NaOH was added to the original system to insure a sufficiently high initial dispersion of the soap particles (as indicated by the optimum drop number), to prevent aggregation of the particles and their deposition under the influence of gravity. If Brownian movement is maintained up to a certain concentration of NaCl, subsequent adsorption of Na ions causing reduction of negative charge and change of surface tension results in such a distortion of the individual particles that they coalesce simultaneously with one another to form a jelly-like structure containing water in the interstices.

The close similarity in the antagonistic curves of $CaCl_2$ and NaCl and other salts having a more readily adsorbed anion in such widely diversified systems as living protoplasm, the blood clot emulsions, soap jellies and films, and soap and lipoid suspensions, and the common limiting concentrations at which individual salts like NaCl and $CaCl_2$ produce inhibiting effects in all these systems, concentrations which differ entirely from those at which similar effects may be observed on proteins, suggest the probability that protoplasmic equilibrium and the formation of reversible protoplasmic jellies are dependent upon what may be designated as an imperfect reversal of phase relations promoted by the action of antagonistic electrolytes on interfacial films of soap and lipoid just as in reversal of equilibrium in the case of emulsions of oil and water.

ELECTROLYTES AND ANAPHYLAXIS.

By G. H. A. CLOWES.

Ca salts appear to counteract sensitization phenomena by diminishing the permeability of the tissues to water. The fact that Ca salts, owing to their aggregating effect on soap films, may be used to counteract increased permeability resulting from adsorption or interaction of negative ions, and that the ratios in

which various salts having readily adsorbed anions are antagonized by salts of Ca are approximately the same in such widely diversified systems as marine organisms, living mice, blood coagulation, complement hemolysis, soap and lipoid suspensions, and drop systems,[3] coupled with the drop in blood coagulation in anaphylaxis, suggests the probability that the antigen-antibody combination affords a link between water and some non-aqueous phase in the protoplasmic film whereby the latter is subjected to the action of readily adsorbed anions already present in the system with a resulting sudden increase in permeability beyond those limits within which normal protoplasmic function may be maintained.

Support for this contention may be found in the well known surface tension effects and the rôle played by electrolytes in immune phenomena; and a practical analogy is to be found in experiments carried out by means of the drop method, demonstrating that water containing NaOH or NaOH + NaCl may be passed through neutral oil without causing any change in the drop number, and water or water + NaCl may be passed through oil containing fatty acid without change, but when NaOH and fatty acid are both present in the system either as soap in the water phase or as NaOH in the water phase and fatty acid in the oil phase, a considerable rise in the drop number occurs, and if NaCl is present there is in addition a still further rise.

With a drop system in which the conditions of equilibrium appear to be comparable with those of living protoplasms, a rise from ten to fifty drops may be effected by introducing a given amount of soap into the water phase or NaOH into the water phase and fatty acid into the oil phase, and a further rise to 300 drops may be effected by using physiologically normal salt solution instead of water. The great increase in permeability indicated, far in excess of that required to cause destruction of protoplasm, could only be effected in the presence of both alkali and fatty acid, presumably owing to the affinity of water for NaOH, of NaOH for fatty acid, and fatty acid for oil; and the consequent establishment of chemical or physical contact between the water and oil phases without which the dispersing effect of the readily adsorbed Cl ion could not come into play.

CALCIUM AND PHOSPHORUS IN THE BLOOD OF LACTATING COWS.

By EDWARD B. MEIGS and N. R. BLATHERWICK.

(From the Bureau of Animal Industry, United States Department of Agriculture, Washington.)

Calcium, nitrogen, total phosphorus, and lipoid and inorganic phosphorus have been determined in the blood and plasma of cows and heifers of various ages, on various rations, and at various stages of pregnancy and lactation. The results are still highly incomplete, but are sufficient to indicate certain relations.

The calcium content of cows' plasma has been found to be surprisingly constant. It is somewhat over 0.011 per cent in very young heifer calves, and falls off gradually with increasing age until it reaches about 0.010 per cent toward the end of the first year, from which point it remains nearly constant. Our results indicate that it is not appreciably altered by pregnancy, lactation, or by considerable changes in diet. Our highest figure for plasma calcium has been 0.0114 per cent, and our lowest 0.0086 per cent.

The total, lipoid, and inorganic phosphorus contents of normal plasma are all extremely variable. In our experiments the total phosphorus has varied from 0.0085 per cent to 0.0178 per cent; the lipoid phosphorus, from a little under 0.002 per cent to a little over 0.008 per cent; and the inorganic phosphorus, from about 0.004 per cent to about 0.008 per cent. The lipoid phosphorus has been found lowest in very young calves, highest in cows at about the 6th month of lactation, and intermediate in farrow heifers from 1 to 2 years old, and in pregnant cows at the end of the previous lactation period. The inorganic phosphorus has been found highest in young calves, and is likely to be low in mature cows just after calving. It seems to depend to some extent on the amount of grain fed.

In numerous samples of plasma, the total, lipoid, and inorganic phosphorus contents have been independently determined, and it has always been found that the sum of the lipoid and inorganic fractions is equal, within the limits of experimental error, to the total.

Our nitrogen results indicate an increase in the ratio, protein:

water, in plasma with increasing age, and some individual variation in this ratio; but little change as the result of pregnancy, lactation, or change in rations.

STUDIES IN THE MECHANISM OF ABSORPTION FROM THE COLON

By SAMUEL GOLDSCHMIDT AND A. B. DAYTON.

(From the Hunterian Laboratory of Experimental Pathology, Johns Hopkins Medical School, Baltimore.)

Sodium chloride solutions of concentrations above 1.2 per cent when placed into the colon of the dog cause a passage of water into the colon contents. At the same time chlorides are passing into the blood. There is an immediate drop in the concentration of chlorides. The Δ of the colon contents shows an immediate decrease of osmotic pressure. The concentration of chlorides and the Δ at the point where the volume ceases to increase is above the concentration of chlorides and the Δ of the blood.

The concentration of chlorides finally comes to an equilibrium with the chlorides of the blood. An increase of chlorides in the blood causes a readjustment of this equilibrium.

The Δ of the colon contents is not accounted for by the chlorides; hence "other substances" have diffused into the colon from the blood.

Chlorides may be withdrawn from the blood until the concentration in the colon reaches 0.12 to 0.17 per cent, when there is a reabsorption of the chlorides; nevertheless the concentration of chlorides in the colon increases to blood level. The colon is therefore not an "irreciprocal membrane." It is essentially a permeable membrane towards sodium chloride solutions.

A MODIFICATION OF FOLIN'S METHOD FOR THE ESTIMATION OF CREATININE IN BLOOD.*

By THEODORE KUTTNER.

(From Mt. Sinai Hospital, New. York.)

The modified method suggested reduces the disturbing factor of the yellow color of the picric acid, because of the smaller amount of this substance used than in the original Folin method.

*Read by invitation.

For similar amounts of blood Folin's method requires thirty-three times as much picric acid as in the new procedure which depends upon removal of the proteins before the addition of the picric acid. Details of the procedure will be published shortly.

A QUICK TITRATION METHOD FOR DETERMINING SMALL AMOUNTS OF URIC ACID.

By J. LUCIEN MORRIS.

(From the Laboratory of Biological Chemistry, Washington University, St. Louis.)

Precipitation of uric acid as zinc urate is used as the basis of separation of uric acid from other substances in body fluids which would interfere with a subsequent permanganate oxidation. The method is described in detail for urine but has application as well to blood. Albumin is removed by treatment either with trichloroacetic acid or *m*-phosphoric acid. An aliquot part of albumin-free filtrate is added to a special solution of sodium lactate, sodium acetate, and acetic acid, and the phosphates and ammonia are removed by precipitation at room temperature with uranium acetate. The solution obtained after filtering or centrifuging will allow its uric acid content to be removed completely as zinc urate upon the successive addition of zinc acetate and sodium carbonate. Centrifuging for a minute separates the precipitate and the liquid may be poured off. Solution of the precipitate is obtained by the addition of acetic acid and a re-precipitation with sodium carbonate assures the absence of substances which might interfere with the permanganate titration. Solution of the precipitate in acetic acid, heating to 80°C., and adding phosphoric acid until zinc phosphate dissolves are the conditions which immediately precede the titration with 0.005 N potassium permanganate. Use of sulfuric acid gives a figure about 10 per cent higher, due probably to purines which are precipitated with zinc but which are not oxidized with permanganate in acetic acid solution.

NOTE ON A TITRATION METHOD FOR DETERMINING MINUTE QUANTITIES OF ACETONE.*

By ROGER S. HUBBARD.

(From Washington University, St. Louis.)

The solution of iodine used in the regular Messinger determination of acetone is diluted in 3 per cent potassium iodide, and used for the determination of minute quantities of acetone. 15 gm. of potassium iodide are dissolved in water in a 500 cc. volumetric flask, appropriate amounts of the stock iodine solution added, and made to volume. From the stock solution of approximately 0.1 N concentration (1 cc. equals 1 mg. of acetone) 0.01 N and 0.002 N solutions are so prepared. Thiosulfate solutions of corresponding strength are easily prepared, and are fairly stable.

In carrying out the determination the acetone solution is distilled to a volume of 250 cc. and 50 cc. are taken for a determination. 40 cc. of 0.01 N iodine solution are added, followed by 1 cc. of 10 $\times$ N NaOH. The solution is shaken, and allowed to stand 10 minutes. It is then acidified with H_2SO_4, and the excess of iodine titrated with 0.01 N thiosulfate solution, using 1 to 2 cc. of 0.25 per cent starch as indicator.

If the amount of acetone is less than 0.2 mg., the procedure is repeated, using 10 or 20 cc. of 0.002 N iodine solution, and titrating with 0.001 N thiosulfate. A blank should be run on the NaOH used. The iodine solution is permanent for 4 or 5 days, and thiosulfate solution for 48 hours. Amounts of acetone as small as 0.01 mg. may be accurately determined by this method

THE ALLEGED NINHYDRIN REACTION WITH GLYCEROL, ETC.

By VICTOR JOHN HARDING.

(From the Biochemical Laboratory, McGill University, Montreal.)

The author found that in following the work of Halle, Loewenstein, and Přibřam on the production of a ninhydrin reaction by glycerol, speoimens of glycerol of different origin gave different results. The production of a positive ninhydrin reaction with glycerol is believed to be due to the presence of nitrogenous impurities.

*Read by invitation.

THE USE OF HEXAMETHYLENETETRAMINE AS A RENAL FUNCTIONAL TEST, AND ITS COMPARISON WITH OTHER FUNCTIONAL TESTS.*

By W. G. LYLE.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

A method for the quantitative determination of urotropin in the urine has been described by Falk and Sugiura.[4] They found that if 15 grains of urotropin were given to a person with normal kidney function, and the urine was collected for 12 hours, they were able to recover from 40 to 80 per cent of the urotropin unchanged; on the other hand, if the patient was suffering from nephritis, little or no urotropin was excreted. It was suggested that this fact should be utilized as a test for the functional activity of the kidney. A comparison of this method with other functional kidney tests was undertaken.

In general it may be stated that in cases where the clinical symptoms point undoubtedly to nephritis nearly all the functional tests agree fairly well. In early cases, however, where the diagnosis is in doubt, urotropin is of value. The failure of the kidney to excrete the urotropin molecule is a very early symptom of interference with its function. It is of interest to note that this phenomenon coincides closely with the finding of an increased amount of uric acid in the blood, and with the changes in the kidney function, as shown by the Mosenthal test meal, especially those cases showing a marked increase in the volume of night urine. In this type of case we have frequently found a good, or even high dye test and a normal non-protein nitrogen and urea. Whether some of these cases will develop a true nephritis remains to be seen, but that there is present some disturbance of kidney function is evident.

In conclusion, from our work with these tests, one fact stands out clearly,—that it is unwise to base a diagnosis on the result of one or two tests as there are cases in which the uric acid in the blood is normal while the urotropin, Mosenthal meal, and the dye test show kidney disturbance. On the other hand, cases of gout may show a high uric acid content in the blood, while the

*Read by invitation.

[4] Falk, K. G., and Sugiura, K., *J. Pharm. and Exp. Ther.*, 1917, ix, 241.

other tests are normal. The advantage of urotropin is that the test is easily carried out and gives the patient no inconvenience. We therefore recommend that in a suspected case the patient should be given 15 grains of urotropin, dissolved in a glass of water, shortly before the evening meal, at which time fluid should be taken to the amount equivalent to two more glasses of water; a 12 hour specimen should be collected and examined for urotropin; if none is found, or a small amount, the other tests should be carried out.

THE CHOLESTEROL CONTENT OF THE BLOOD IN VARIOUS HEPATIC CONDITIONS.*

BY M. A. ROTHSCHILD AND J. FELSEN.

(From the Department of Physiological Chemistry, Pathological Laboratory, Mount Sinai Hospital, New York.)

In obstructive icterus the cholesterol content of the blood is markedly elevated and bears a definite relationship to the intensity of the icterus. In conditions such as cirrhosis, acute yellow atrophy, pernicious vomiting of pregnancy, or extensive carcinomas of the liver, the cholesterol content of the blood is not increased and is usually reduced. The cholesterolemia is thus not proportionate to the intensity of the icterus. In other forms of icterus, as seen in Banti's disease, Gaucher's disease, splenomegaly with icterus, and pernicious anemia and other blood diseases, there is no increase in the blood cholesterol, and in most of these conditions the amount present is decidedly less than normal.

THE USE OF PANCREATIC VITAMINE IN CASES OF MALNUTRITION.

BY WALTER H. EDDY.

(From the Department of Pathology of the New York Hospital.)

The report covers the preliminary study of the use of pancreatic vitamine in cases of marasmus. Up to the present time the treatment has been administered to eleven cases, seven of which form the basis of the present paper; the other four are not sufficiently advanced to supply data at this time.

* Read by invitation.

The treatment consists in feeding children 2 gm. per day of Lloyd's reagent which has been activated by pancreatic vitamine after the method of Seidell. Previous work had demonstrated that 50 gm. of Lloyd's reagent shaken with 1 liter of the water-soluble portion of an alcohol extract of pancreas remove from that volume its growth-stimulating power. In these experiments 1 gm. of Lloyd powder contains the vitamine from 54 gm. of pancreas. (This assumes that the removal of the vitamine is quantitative.) The powder is administered by mixing 1 gm. with a feeding of cereal. No difficulties have arisen in getting the children to take this material. The success of the experiment is judged by noting whether growth develops after administration of the vitamine, no other change being made in the diet.

The results suggest at least the possibility of stimulating assimilation by the artificial feeding of pancreatic vitamine. The dosage and the conditions favorable and unfavorable to its effectiveness are under investigation.

THE URIC ACID CONTENT OF THE BLOOD OF THE NEW-BORN.

By F. B. KINGSBURY, J. P. SEDGWICK, and L. J. ROBERTS.

Uric acid determinations in the blood of the new-born showed that the concentration of uric acid varied from 1.8 to 3.5 mg. per 100 gm. of placental blood, and from 2.7 to 4.9 mg. per 100 gm. of blood drawn from the new-born 8 to 120 hours later. The uric acid content in the case of a new-born 8 hours old was 4.9 mg. per 100 gm. of blood. At the end of 10 days this value had dropped to 1.4 mg. In the case of another baby the placental blood contained 1.9 mg. which rose to 4.0 mg. 46 hours later, and at the end of 9 days was 3.8 mg. Both placental blood and blood taken a few hours after birth contain more uric acid than that of the normal adult. A definite increase after birth is shown and this occurs during the same period of time that uric acid infarcts in the kidney are known to be formed.

THE PARTITION OF NON-PROTEIN NITROGEN IN THE BLOOD OF FRESH WATER FISH.

BY D. WRIGHT WILSON AND EDWARD F. ADOLPH.

(From the Laboratory of Physiological Chemistry, Johns Hopkins University, Baltimore, and the United States Fisheries Biological Station, Fairport, Iowa.)

The whole blood and plasma from several species of fresh water fish were analyzed for non-protein nitrogen, urea, ammonia, amino nitrogen, uric acid, creatinine, and creatine. The fish studied were the sturgeon, gar, carp, croppie, catfish, and sheepshead. The total non-protein N was lower in the ganoids (29 to 31 mg. per 100) than in the teleosts (42 to 59 mg.). The plasma contained less than the whole blood in all cases. The urea nitrogen was unusually low in most specimens, varying from 1.1 to 16 mg. per 100 cc. of whole blood and from 0.4 to 13 mg. per 100 cc. of plasma. The urea content of the plasma was less than whole blood in all cases. The amino nitrogen fraction made up from 60 to 81 per cent of the total non-protein nitrogen of the whole blood, and from 33 to 45 per cent of the total non-protein nitrogen of the plasma. The ammonia, uric acid, and creatinine contents of whole blood were similar to the other values reported for fish. The creatine content of plasma was higher than that of the corpuscles. The considerable variations in the composition of the blood of fish and higher animals suggest differences in metabolism.

THE DISTRIBUTION OF CREATININE AND CREATINE BETWEEN THE CORPUSCLES AND PLASMA OF THE BLOOD.

BY ANDREW HUNTER AND WALTER R. CAMPBELL.

(From the Department of Pathological Chemistry, University of Toronto.)

In forty-eight specimens, two of which came from rabbits and the remainder from human subjects, the creatinine contents of the whole blood and of the plasma were separately determined. Creatine estimations were similarly carried out in forty-two cases, in thirty-three of which the measurement of corpuscular volume enabled us to calculate the concentration of both substances in the corpuscles also. These data were supplemented in twenty-

six cases by a determination of total and preformed creatinine in the urine excreted at the time of the observation.

The single generalization which we can draw in the meantime from our results is that the concentration of both creatine and creatinine is considerably lower in plasma than in whole blood, so that, bulk for bulk, the corpuscles are much richer in these substances than the plasma. The *plasma* creatinine of normal individuals was always less than 1 mg. per 100 cc.; in all but two it fell between 0.72 and 0.92, the average being 0.82. The plasma creatine in the same subjects ranged from 0.94 to 3.5; but in two-thirds of them it lay between 1.4 and 1.7, and the average for human individuals was 1.6. The creatine and creatinine content of the *corpuscles* varied within rather wide limits, but the second was always present in much greater quantity than the first.

When creatinine is retained, as in the nephritic cases, it appears generally to accumulate in the corpuscles as well as the plasma. We have, nevertheless, encountered some cases in which the plasma creatinine seemed to have increased out of proportion to that of the whole blood, and others in which blood containing an abnormally large quantity of creatinine yielded a plasma with the normal amount. These occasional findings encourage us to hope that the plasma creatinine, as distinct from the blood creatinine, may prove to have a diagnostic value of its own.

The concentration of creatine in the plasma does not appear to bear any relation to the occurrence of creatine in the urine. We have obtained no indication of the existence of a threshold for creatine.

Observations on parturient women seem to indicate that there is a ready interchange of both substances between the mother and the fetus. The concentration of each is nearly the same in both bloods, and especially in the plasmas. Where a difference exists it is almost always in the direction of a somewhat higher concentration in the placental blood. In the majority of our cases the creatinine content of maternal and placental blood was above the normal.

The results reported are of a preliminary character only, and the work is being continued along each of the lines suggested.

THE EFFECT OF ARTIFICIAL RESPIRATION ON GLYCEMIA.

By I. S. KLEINER and S. J. MELTZER.

(From the Department of Physiology and Pharmacology of The Rockefeller Institute for Medical Research.)

The hyperglycemia which is produced by an intravenous injection of magnesium sulfate was shown in a previous report[5] not to be due to asphyxia, as had been suggested.[6] In those experiments artificial respiration had been given in order to prevent asphyxia, and, nevertheless, a marked hyperglycemia had been caused by the magnesium injection. It occurred to us later that possibly these results might have been due simply to the artificial respiration, since Henderson and Underhill[7] have reported that acapnia may produce an increase in the percentage of blood sugar. We have therefore determined the influence of artificial respiration upon the blood sugar concentration.

Ten dogs were used in this work. Under local anesthesia a cannula was inserted in a femoral artery and a tracheal cannula introduced for artificial respiration. In five experiments there were 20 or 21 interruptions of the air current per minute, and in five 40 to 44 per minute. Artificial respiration of the ordinary type, but with comparatively high air pressure, was administered for 1 hour. In none of the experiments was there any marked increase in the blood sugar, 0.04 per cent being the greatest increase in any. In five out of the ten experiments there was no increase whatever.

It is thus evident that vigorous artificial respiration for 1 hour causes no appreciable effect upon the percentage of sugar in the blood and therefore the hyperglycemia present when $MgSO_4$ is injected cannot be the result of an overventilation by the artificial respiration.

THE NATURE OF THE BLOOD SUGAR.

By HUGH McGUIGAN.

(From Northwestern University Medical School, Chicago.)

The work is based mainly on the known fact that picric acid does not interfere with the determination of blood sugar by

[5] Kleiner, I. S., and Meltzer, S. J., *J. Biol. Chem.*, 1916, xxiv, p. xx.
[6] Underhill, F. P., and Closson, O. E., *Am. J. Physiol.*, 1906, xv, 321.
[7] Henderson, Y., and Underhill, F. P., *Am. J. Physiol.*, 1911, xxviii, 275.

Fehling's solution. The results, however, were corroborated by other methods. If the filtrate from the picric acid is analyzed directly it is found to contain on the average about 25 per cent of the total blood sugar. When, however, the picric acid filtrate is boiled with 4 per cent acetic acid for 2 minutes the sugar yielded rises on the average four times. The results indicate a hydrolysis probably of a dextrin. The dialyzed serum gives the same results. Ether anesthesia changes the ratio of the polysaccharide (?) to the dextrose from 4:1 approximately to 1:1. In severe diabetes the ratio is approximately 1:1.

There is a possibility of an interfering substance in the picric acid filtrate which may hold the cuprous oxide in solution, but we have been unable to find such a body. Consequently we think that any one method of blood sugar analysis is insufficient, that the ratio of the free to the combined or polysaccharide should be determined, and that the ratio of the two is of significance in diabetes. The polysaccharide or combined form corresponds to what has been called virtual sugar.

THE ENZYMES OF THE TUBERCLE BACILLUS.

By H. J. CORPER and H. C. SWEANY.

(*From the Municipal Tuberculosis Sanitarium of the City of Chicago.*)

Tubercle bacilli both of the human and bovine variety possess autolytic enzymes, as indicated by the liberation at incubator temperature of non-coagulable nitrogen, and of amino-acid α-nitrogen after the bacilli have been killed by toluene and chloroform. The bacilli themselves, or autolysates therefrom, also possess a trypsin-like enzyme capable of splitting proteins in alkaline solution, a weak pepsin-like enzyme capable of splitting proteins in acid, an erepsin-like enzyme capable of decomposing peptone in acid solution, a nuclease capable of acting upon nucleic acid, and a urease capable of decomposing urea. The tubercle bacilli, or autolysates therefrom, do not possess enzymes capable of hydrolyzing starch or inverting saccharose at incubator temperature, at least not to an extent capable of demonstration by the delicate Lewis and Benedict colorimetric picramic acid method. Autolysates from tubercle bacilli do not pr
zymes capable of digesting elastic tissue prepared from

lung, or connective tissue prepared from tubercles, at least not by the methods used for demonstrating these enzymes. The demonstration of the latter enzymes of necessity had to be by indirect methods of attack and are, therefore, open to criticism.

A STUDY OF FOOD FISHES. THE COMPLETE ANALYSIS OF TWENTY COMMON FOOD FISHES, WITH ESPECIAL REFERENCE TO A SEASONAL VARIATION IN COMPOSITION.

By E. D. CLARK and L. H. ALMY.

(*From the Food Research Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

In the present investigation specimens of twenty different species of fish were analyzed, some only once and others twice or more times during the year. In general, there is little variation in the amount of nitrogenous and ash constituents, but fat is the great variable, its amount depending upon the season, age of fish, and relation to time of spawning and feeding. The typical migratory fish, like shad, bluefish, weakfish, and mackerel, sometimes show variations from less than 0.5 per cent to over 16 per cent, depending upon the factors mentioned above. On the other hand, typical bottom fish, like cod, haddock, and flounders, have a minimum amount of fat which varies but slightly compared with surface and migratory species. Analysis of individuals taken from the same school of fish also show variations in fat content from 1 to 8 per cent in the case of weakfish. Some species, like shad and salmon, are fattest in the spring, while others, like bluefish and butterfish, are at their best in the fall just after the active feeding period. In the case of shad, the percentage of fat decreased from a maximum of over 14 per cent in April before spawning to a minimum of less than 3 per cent in June when the fish were spent after spawning.

In the case of the nitrogenous constituents of fish there is much less variation than in the fat. The coagulable and hot-water-soluble constituents (gelatin-like substances) vary somewhat with the species but change little from spring to autumn or winter. There were striking differences in the percentages of ammonia nitrogen, but it is not certain whether they were due to individual or species differences or to the varying leaching effect of water from melting ice in which the fish were packed for shipment.

A NOTE ON CERTAIN CHEMICAL CHANGES IN SHUCKED OYSTERS UNDER REFRIGERATION, AND METHODS OF DETECTING THESE CHANGES.

By E. D. CLARK and L. H. ALMY.

(*From the Food Research Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

The work reported deals with chemical methods of supplementing bacterial counts in the analysis of oysters. Preliminary experiments showed that freshly shucked oysters standing at temperatures above the freezing point yielded regularly increasing percentages of ammoniacal nitrogen by aeration, amino-acid nitrogen, and reducing substances.

Larger scale studies were then made upon lots of oysters held at ice box temperatures (45° F.) and in cold rooms at 40° F. and 34° F., respectively. All these temperatures are regularly employed in the commercial handling and transportation of oysters, either in car lots or small packages. To simulate commercial practices in handling and transporting oysters, samples were held at different temperatures for various periods of time up to 15 days. During storage the ammoniacal nitrogen showed the most marked increases, but the low temperature of 34° F. had a great inhibiting effect on the production of ammonia. The amino-acid nitrogen and reducing substance (probably sugar) did not show increases of the same magnitude as ammonia because they may have been assimilated by bacteria, while ammonia is an end-product of bacterial metabolism. The amino-acid nitrogen was determined by Van Slyke's gasometric method and by Sörensen's formol titration method, both of which procedures gave satisfactory and concordant results on oyster extracts. The reducing substances were estimated by Benedict's quantitative method for sugar, using alkaline copper solution.

In general, the lower temperatures greatly retarded the formation of ammonia during storage, but apparently did not affect the other changes to as great an extent, possibly because increases in amino-acid nitrogen and reducing substances were due to enzymes which were much less retarded by low temperatures than was the growth of bacteria concerned in the production of ammonia. The reducing substance may have been glucose

arising from the hydrolysis of glycogen in the oyster tissues. Living oysters, or those suddenly killed by plunging into boiling water, contained hardly a trace of reducing substance. As is the case with all perishable substances, decomposition in oysters is not prevented by low temperatures but is merely retarded, especially at temperatures near the freezing point.

PRELIMINARY OBSERVATIONS ON THE REGULATION OF THE CALCIUM EXCRETION IN THE DOG.

By MAURICE H. GIVENS.

(From the Sheffield Laboratory of Physiological Chemistry, Yale University, New Haven.)

A dog weighing 13.3 kilos has been maintained in nitrogenous equilibrium over a period of 60 days, during which time the calcium balance has been negative. This was brought about by *feeding on a meat, lard, cracker meal diet. When for the cracker* meal dried skimmed milk and sucrose were substituted, on the basis of nitrogen and calorie equivalent, there was an approach toward a calcium balance. The dried skimmed milk diet contained six and a half times as much calcium as the meat, cracker meal ration.

When sodium bicarbonate (0.5 gm. per kilo) was added to the meat, cracker diet, no effect was produced on the calcium output. No effect was observed on the addition of bicarbonate to the dried milk feed.

On the other hand, when hydrochloric acid (0.11 gm. per kilo) was added to the meat, cracker diet, there was a prompt increase in the excretion of calcium by way of the kidneys, presumably through diversion from the intestinal path. When the acid was superimposed upon the high calcium diet there was likewise an increased urinary excretion of calcium though not at the expense of the fecal output.

The reported effect of alkali bicarbonate upon the calcium output during various levels of calcium intake has been confirmed on a second dog. These studies are being continued.

THE INFLUENCE OF ACID AND ALKALI ON THE ABSORPTION OF GLUCOSE FROM THE INTESTINE.

By JOHN R. MURLIN.

(From the Physiological Laboratory of Cornell University Medical College, New York City.)

Loops of the small intestine of chloretonized dogs were measured off so as to be as nearly equal in length as possible, washed out with warm Ringer's or Tyrode's solution, and then filled with equal quantities of glucose solutions, one having a certain strength of alkali measured as anhydrous sodium carbonate, and the other an equivalent amount of hydrochloric acid. The solutions were permitted to remain in the intestine for from 30 to 165 minutes, then withdrawn, and titrated for sugar with Benedict's solution. Out of nearly two dozen experiments only two failed to show more absorption from the alkaline medium than from the acid, and in one of these there was sign of leakage through the ligatures.

The following may be cited as examples.

Dog No.	Glucose given.	Strength of alkali.	Absorption per minute.		
			Alkaline loop.	Acid loop.	Length of time.
	gm.		*mg.*	*mg*	*min.*
II	2.97	0.3 per cent.	20	10	34 and 37
III	2.94	0.5 " "	20	12	120
IV	2.14	0.3 " "	13	10	165
VI	1.90	0.3 " " in both loops.	14	15	90
A	2 per cent.	0.1 N	0.36	0.63	30
B	2 " "	0.2 "	0.035*	0.166*	45

* Per cm. length. The loops were not equal in length.

In general, the greater the concentration of the acid and alkali, the greater was the difference in absorption between the two loops. In several instances the mucous lining of the alkaline loop was ground and hydrolyzed with hydrochloric acid, but no reducing substance was found.

The explanation of the difference seems to be the different effects of alkali and acid on protoplasm, the one tending to increase permeability, the other to decrease it.[8]

[8] Osterhout, W. J. V., *J. Biol. Chem.*, 1914, xix, 335.

AN EXPERIMENTAL STUDY OF LIPOLYTIC ACTIONS.

By K. GEORGE FALK.

(*From the Harriman Research Laboratory, Roosevelt Hospital, New York.*)

The inactivation of esterase and lipase from castor beans by acids, bases, neutral salts, alcohols, acetone, esters, and heat led to the hypothesis that the lipolytically active grouping is the tautomeric enol-lactim form of the peptide linking which becomes inactive on rearrangement to the keto form. Experiments testing this view resulted in the production of lipolytically active substances by the action of alkali on castor bean globulin, casein, and gelatin. Further confirming evidence was obtained on studying the ester-hydrolyzing action of glycine, glycyl-glycine, and hippuric acid at different hydrogen ion concentrations. Summarizing, it may be said that, given a definite chemical grouping the nature of which has been indicated, and which may be present in different classes of substances, certain definite lipolytic actions will result.

A METHOD FOR THE SEPARATION OF THE DIETARY ESSENTIAL, "FAT-SOLUBLE A," FROM BUTTER FAT.

By E. V. McCOLLUM, N. SIMMONDS, and H. STEENBOCK.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

Fat-free milk, when included in a diet consisting otherwise of purified food substances, promotes growth and prevents decline of animals in a manner which indicates that it still contains the fat-soluble essential in appreciable amount. This led us to suspect that it is appreciably soluble in water. Experiments have been conducted in which butter fat was employed which had been melted and thoroughly agitated with twenty successive portions of water. After this treatment it is no longer effective in inducing growth when fed with suitably constituted diets. Change to unwashed butter fat was followed by prompt resumption of growth.

ANTINEURITIC SUBSTANCES FROM EGG YOLK.

By H. STEENBOCK.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

By means of neutral solvents there was prepared a water-acetone-soluble fraction from egg yolk which in small doses by intraperitoneal injections was able to cure a pigeon suffering from polyneuritis. The antineuritic principle was found stable to concentrated hydrochloric acid at 98° and to concentrated alkalies at room temperature, but readily destroyed by dilute alkalies at the boiling temperature. Phosphotungstic acid precipitated it incompletely. It was not adenine.

THE ISOLATION OF STACHYDRIN FROM ALFALFA HAY.

By H. STEENBOCK.

(*From the Laboratory of Agricultural Chemistry, University of Wisconsin, Madison.*)

l-Stachydrin has been isolated in pure form and as the hydrochloride from the phosphotungstic acid fraction of the water-soluble constituents of alfalfa hay. For its identification the picrate, chloroplatinate, aurate, methyl ester, and methyl ester chloroaurate were prepared and found characteristic. The optical activity was found slightly below the reported value though of the same sign. Both the Kossel and the Van Slyke methods when applied directly to alfalfa hay nitrogen give erroneous values for the diamino-acids, the error in the first case falling upon the lysine fraction and in the second case upon the histidine fraction.

A METHOD FOR THE IDENTIFICATION OF PHENYLALANINE-URAMINO-ACID IN THE PRESENCE OF UREA AND AMINO-ACID.*

By ALICE ROHDE.

So far it is undetermined whether uramino-acids exist preformed in the body fluids or whether when isolated they are the products of the interaction of urea and of amino-acid present.

* Read by invitation.

Large intravenous doses of phenylalanine were administered to cats under urethane anesthesia, the urine was collected from bladder cannulas, and the urea completely destroyed as secreted by urease. After extraction of the urea-free material with acetic ether the aqueous residues left on distillation with steam were analyzed for traces of uramino-acid by the gasometric method for the determination of amino nitrogen. No phenylalanine-uramino-acid could be detected. The determination of phenylalanine-uramino-acid added to urine and Jack bean extract is approximately quantitative by this method, so that one may expect to identify minute amounts of preformed uramino-acid if present.

THE ABSORPTION OF ALCOHOL AND ITS CONCENTRATION IN THE URINE WHEN INJECTED BY RECTUM.

By T. M. CARPENTER and E. B. BABCOCK.

(From the Carnegie Nutrition Laboratory, Boston.)

Studies were made with four medical students of the absorption of ethyl alcohol when introduced by the rectum. Concentrations of 5, 7.5, and 10 per cent were used and amounts varying from 200 to 1,000 cc. were introduced into the rectum. The average amount of alcohol given was about 25 gm. The solutions were retained from 2 to 5 hours. The unabsorbed alcohol was determined in the washouts by the Nicloux method. The absorption of alcohol was practically complete in all cases, the highest amount unabsorbed found being about 0.5 gm.

The concentration of alcohol in the urine was also determined by collecting the urine in as short periods as possible after the alcohol was introduced into the body either by mouth or by rectum. The maximum concentration was about 0.40 mg. per 1 cc. and appeared between the 1st and 2nd hours after ingestion. It fell to zero in about 5 to 6 hours. In one subject there was a tendency for the alcohol percentage to be higher when it was taken by mouth; in the other the concentrations were about the same irrespective of the method of ingestion.

THE COMPOSITION OF RENAL AND VESICAL CALCULI.

By SAMUEL BOOKMAN.

In the quantitative analysis of fourteen stones, it is proven that the alkalinity of the urine is probably at no time sufficiently strong to dissolve the uric acid and urate stones, neither is it acid enough to cause the solution of oxalate and phosphate stones, which will account for the formation of mixed stones. The percentage composition of these stones exemplifies the change from the acid to the alkaline precipitation, or the reverse, the phosphates and carbonates being precipitated in the alkaline condition, and the urates and uric acid in the more acid reaction, the oxalates in the less acid urines, as well as in the alkaline condition. No doubt the disturbance in the colloid equilibrium plays an important part in this precipitation, but in addition we have the following contributing conditions to reckon with as well: diet, acidity, concentration, inflammation, and bacterial decomposition within the urinary tract.

THE ACTIVE CONSTITUENT OF THE THYROID: ITS ISOLATION, CHEMICAL PROPERTIES, AND PHYSIOLOGICAL ACTION.

By E. C. KENDALL.

(*From the Mayo Clinic, Rochester, Minn.*)

Primary cleavage of thyroid proteins results in acid-insoluble A and acid-soluble B constituents. Separation of the iodine compound from A is accomplished by the solubility of its barium compound in barium and sodium hydroxides. Many successive precipitations, alternated with heating in sodium hydroxide and the action of carbon dioxide, remove impurities, the last traces of which are removed by dissolving in alkaline alcohol and precipitating with acetic acid. The active constituent separates in microscopic needles. It may be precipitated as free base or in salt form. The free base is insoluble in alcohol and contains 65 per cent of iodine. The sulfuric acid salt is soluble in alcohol and contains 60 per cent of iodine. Its molecular weight appears to be 586. The chemical properties depend upon the degree of purification and the presence of other substances. In impure form heating with acid destroys the physiological activity and gives a product resembling Baumann's iodothyrin.

The physiological activity depends upon the amount administered and the susceptibility of the individual. 0.125 to 0.25 mg. daily is sufficient for cretinism. The maximal quantity tolerated by human beings so far tried has been 2 mg. daily. The response is not immediate; 24 to 72 hours may elapse before the physiological effects appear. So called hyperthyroidism symptoms and death follow administration of excessive doses to animals.

NEW BUFFER MIXTURES, STANDARD TUBES, AND COLORIMETER FOR DETERMINING THE HYDROGEN ION CONCENTRATION OF SEA WATER.*

By J. F. McCLENDON.

(From the Physiological Laboratory of the University of Minnesota, Minneapolis.)

Two stock solutions were used: (1) 0.3 M boric acid containing 2.25 per cent NaCl, and (2) 0.075 M borax containing 1.9 per cent NaCl, and mixed in thirty different proportions that may be estimated by plotting from data in the following table. The first column gives the percentage of the boric acid solution, the second that of the borax solution, the third the P_H of sea water of 13.9 gm. Cl per liter, the fourth the P_H of sea water of 17.7 gm. Cl per liter, and the fifth the P_H of sea water of 21.5 gm. Cl per liter. The tubes of borate mixtures and sea water have been calibrated fifty times by means of the hydrogen electrode, and recalibrated by the same means at Princeton University.

79.5	20.5	7.50	7.45	7.40
72	28	7.70	7.65	7.60
68	32	7.80	7.75	7.70
60	40	8.00	7.95	7.90
37	63	8.50	8.45	8.40
8	92	9.00	8.95	8.90
1	99	9.10	9.05	9.00

Thymolsulfophthalein was used as indicator from P_H 7.9 to 9 and *o*-cresolsulfophthalein from P_H 7.5 to 8.3, so as to form 0.001 per cent solution in both buffers and sea water. The buffers were sealed in "Nonsol" tubes of 24 mm. bore and the sea water

* Read by title.

was placed in open tubes of the same bore, and compared in a colorimeter having the lenses of a stereoscope placed in contact so as to bring the centers of the tubes together in a sharp line when viewed from the side with one eye 6 inches from the lenses. Tables were prepared for determining the CO_2 tension, CO_2 content, O_2 content, and heat given off in the respiration of marine animals.[9]

THE INFLUENCE OF TEMPERATURES ABOVE FREEZING ON THE CHANGES IN CHEMICAL COMPOSITION, BACTERIAL CONTENT, AND HISTOLOGICAL STRUCTURE OF THE FLESH OF THE COMMON FOWL.

By M. E. PENNINGTON, J. S. HEPBURN, E. Q. St. JOHN, and E. WITMER.

(*From the Food Research Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Chickens were killed and picked, and were kept at a temperature of approximately 0°C. for 24 hours, in order to remove the animal heat. Some birds were then analyzed as "freshly killed chickens;" others were kept in the room (average temperature 23.9°C., maximum holding period 4 days), or in a house refrigerator (temperature 7.2–12.8°C., maximum holding period 7 days), or in a mechanically refrigerated "chill room" (temperature 0°C., maximum holding period 3 weeks), and were then analyzed. A number of analyses were made, both of freshly killed birds and of birds kept at each of the temperatures stated. While the total nitrogen content of the muscle remained unchanged, the partition of the nitrogen underwent changes at all three temperatures of holding. Thus the total nitrogen of the aqueous extract invariably increased in the chickens kept in the room, the increase being a direct function of the period of holding; in the refrigerator this fraction of the nitrogen at times either increased or decreased, depending on the period of holding; in the chill room it decreased during the first 2 weeks of holding, then increased. At all three temperatures of holding, the amino-acid and basic nitrogen increased at the expense of the protein nitrogen, usually in a progressive manner. The most striking change in the fat constants

[9] The sealed tubes and colorimeter may be obtained from Hynson, Westcott, and Dunning, Baltimore.

was an increase in the acid value, which became progressively greater as the period of holding lengthened. Both the formation of amino-acid and basic nitrogen, and the increase in the acid value of the fat occurred most rapidly at the temperature of the room, least rapidly at that of the chill room. Frequently the saponification number and the Hehner number of the fat increased or decreased *simultaneously*. The bacteria, which grow under aerobic conditions, increased in both the muscles and the skin at all three temperatures of holding; the increase was enormous in the chickens kept in the room, and was less marked, though distinct, in those kept in the refrigerator, and in the chill room; the increase usually became greater as the period of holding lengthened. Organisms which liquefy gelatin were comparatively few in number, even in the chickens which had been kept in the room, and in which the proteolysis had become most advanced. The muscle cells changed in width at all three temperatures of holding. A pronounced swelling or increase in width occurred at the temperature of the room, and a slight increase in width at the temperature of the refrigerator. A shrinkage or decrease in width of the muscle cells—usually progressive—took place at the temperature of the chill room. At all three temperatures, room, refrigerator, and chill room, rupture of the muscle fibers and exudation of the cell contents occurred; the muscle, its nerves and blood vessels, and the connective tissue became altered in histological structure and in the staining power of their various elements; these changes in structure and in affinity for stains depended in part on the temperature, and in part on the time of holding.

The changes which occurred during holding in the chill room for 3 weeks were about equal to those occurring during holding in the house refrigerator for 5 to 7 days. The changes which took place during holding in the house refrigerator for 5 days were less than those taking place during holding in the room for 2 days.

THE INFLUENCE OF TEMPERATURES BELOW FREEZING ON THE CHANGES IN CHEMICAL COMPOSITION, BACTERIAL CONTENT, AND HISTOLOGICAL STRUCTURE OF THE FLESH OF THE COMMON FOWL.

By M. E. PENNINGTON, J. S. HEPBURN, E. Q. St. JOHN, and E. WITMER.

(*From the Food Research Laboratory, Bureau of Chemistry, United States Department of Agriculture, Washington.*)

Chickens were killed and dry picked and kept at 0°C. for 24 hours to remove the animal heat. They were next frozen solid by holding for 48 hours in a "sharp freezer" at a temperature not higher than −14.4°C., then were stored in mechanically refrigerated "freezers" at a temperature of −9 — −13°C. for definite periods of time, the maximum period being 2 years. Every 3 or 4 months, several samples were taken from the freezer and analyzed. The "freshly killed chickens" of the preceding research were taken as the fresh standards with which to compare these hard frozen chickens. The muscular tissue showed a progressive desiccation, or loss of water, while the chickens were held in the freezer. The total nitrogen of the muscle remained unchanged; however, changes occurred in the partition of the nitrogen. Thus the total nitrogen of the aqueous extract increased or decreased, depending on the period of holding. The amino-acid and basic nitrogen showed small but progressive increases; this indicated that a slow but distinct proteolysis had occurred, leading to the accumulation of non-protein nitrogenous compounds at the expense of the protein. The acid value of the fat increased; the saponification number and the Hehner number decreased during the early months of holding, then increased. The hydrolysis of the fat and the digestion of the protein occurred far less rapidly than in chickens held at temperatures above freezing.

The bacteria, which grow under aerobic conditions, decreased in number in the muscle during the freezer storage; the decrease was apparent at the end of 4 months, became more marked as the period of holding lengthened, and was most marked after the birds had been in the freezer for 1 year or longer. Organisms which liquefy gelatin and those which grow in an anaerobic

environment were present only occasionally in the chickens from the freezer; organisms of putrefaction, which grow under anaerobic conditions after their suspension in physiologic salt solution has been kept at 80°C. for 10 minutes, were present still less frequently. The evidence points to an enzymic rather than to a bacteriologic cause for the foregoing chemical changes.

The phenomenon of freezing in itself produced little, if any, change in the width of the muscle cells, as was shown by measurement of sections cut from different pieces, removed from the same muscles before and after freezing the chickens at an average temperature of −11°C. in the "freezer." However, during prolonged holding in the freezer, a progressive shrinkage or decrease in width of the cells occurred. Muscle fibers with a ruptured sarcolemma and exuding cell contents became more numerous as the period of holding lengthened. Characteristic changes took place in the histological structure of the muscle cells, nerve fibers, blood vessels, and connective tissue, and in the affinity of their elements for stains. When chickens were stored in the freezer in closed metallic containers, desiccation was prevented; the other changes were retarded but not prevented. The changes here recorded were most marked *after* the normal commercial period of freezer storage had been exceeded. Both the analyses and organoleptic tests showed that no loss of food value had occurred while the chickens were in the freezer; the birds were still wholesome and nutritious; the only appreciable change was a loss in flavor after holding in the freezer for a period greater than 9 months.

The changes in chemical composition during holding in the freezer for 1 year were about equal to the changes which occurred during holding in the chill room for 3 weeks.

AUXO AMYLASES.

By ELBERT W. ROCKWOOD.

(From the University of Iowa, Iowa City.)

Many amino-acids increase the hydrolytic cleavage of starch by ptyalin. This is true of the α-amino-acids such as glycocoll and tyrosine which result from the hydrolysis of proteins. The accelerating effect is not lost by substituting other radicals for

hydrogen of the amino group, as in hippuric acid. Where the amino group is substituted in the carboxyl the compound has no stimulating effect, as in acid amides including urea. Introduction of the sulfonic radical neutralizes the effect of the amino group, as in sulfanilic acid. The position of the amino group, as in the aminobenzoic acid isomers, does not modify their activity; all stimulate the ptyalin. Compounds of amino-acids, like the proteins, also increase the amylolytic activity of ptyalin. As far as has been determined the pancreatic amylase is similarly affected by the amino-acids. The work is being continued.

A CHEMICAL STUDY OF PROLONGED INANITION.

By R. E. SWAIN.

(From Leland Stanford Junior University, California.)

The subject studied was a tailor by occupation, 30 years of age, who voluntarily subjected himself to a period of starvation which was continued for 60 days. The body weight was about 61 kilos at the beginning of the fast and about 36 kilos at the end. The excreta were collected for analysis from the 40th to the 60th days, and at intervals during this period the body temperature, pulse rate, blood counts, and other clinical data were recorded. The total urinary nitrogen output was maintained at a higher level than that recorded in previous cases of fasting, and shows a rise from the 53rd to the 56th days similar to the premortal rise in lower animals. This rise was followed by a rapid decrease in the daily excretion for the rest of the period. Ammonia is relatively very low. Creatinine was fairly constant until the 54th day when, following the rapid increase in the total nitrogen output and subsequent rapid fall, it decreases in amount to the end of the fast. Creatine is relatively high, exceeding the creatinine in amount during the period of high total nitrogen.

ADENINE-URACIL DINUCLEOTIDE AND THE STRUCTURE OF YEAST NUCLEIC ACID.*

By WALTER JONES and B. E. READ.

*Published in full in *J. Biol. Chem.*, 1917, xxix, 111.

STUDIES OF AUTOLYSIS.

V. THE INFLUENCE OF BILE ON AUTOLYSIS.*

By H. C. BRADLEY and JOSEPH TAYLOR.

THE COMPOSITION OF ADIPOCERE.**

By R. F. RUTTAN and M. J. MARSHALL.

THE USE OF COTTON SEED AS FOOD.†

By THOMAS B. OSBORNE and LAFAYETTE B. MENDEL.

CYSTINE AND THE MINIMUM PROTEIN REQUIREMENT OF THE DOG.

By HOWARD B. LEWIS.

DEMONSTRATIONS BEARING ON COLORIMETRIC CREATININE AND NITROGEN DETERMINATIONS.

By OTTO FOLIN, E. A. DOISY, RICHARD BELL, and W. DENIS.

A NEW TEST FOR THE DETECTION OF OCCULT BLOOD IN STOOLS.‡

By W. G. LYLE.

THE PRODUCTION OF LACTIC ACID BY INTRAVENOUS INJECTION OF ALKALI.

By J. J. R. MACLEOD.

OBSERVATIONS ON THE FATE OF INOSITE IN THE DOG.

By ISIDOR GREENWALD and MORRIS WEISS.

ALKALINITY AND CARBONIC ACID IN SEA WATER.

By L. J. HENDERSON and E. J. COHN.

SOME EXPERIMENTS ON PROTEOLYTIC ACTIONS.‡

By E. M. FRANKEL.

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**Published in full in *J. Biol. Chem.*, 1917, xxix, 319.
†Published in full in *J. Biol. Chem.*, 1917, xxix, 289.
‡Read by invitation.

THE KINETICS OF INVERTASE ACTION.*

By J. M. NELSON and W. C. VOSBURGH.

DEMONSTRATION OF A PERFUSION PUMP.**

By F. B. KINGSBURY.

VIRTUAL SUGAR.

By HUGH McGUIGAN.

THE DETERMINATION OF HEAVY METALS IN PHYSIOLOGICAL TISSUES AND FLUIDS.

By SAMUEL BOOKMAN.

A METABOLISM STUDY OF MANITOU (ALKALINE) MINERAL WATER.

By HELEN I. MATTILL and H. A. MATTILL.

THE REARING OF CHICKS UNDER LABORATORY CONDITIONS WITH ISOLATED FOOD SUBSTANCES.

By M. E. JAFFA and H. A. MATTILL.

THE INORGANIC COMPOSITION OF THORACIC DUCT LYMPH.†

By A. B. MACALLUM.

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*Read by invitation.
**See Kingsbury, F. B., *J. Biol. Chem.*, 1916–17, xxviii, 167.
†Read by title.